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19. ABSTRACT (Continue on reverse if necessary and identify by block number) Anaerobic microbial transformation of monoaromatic hydrocarbons (MAH), chlorinated benzenes (CB), and mixtures of MAH and CB, as well as MAH and chlorinated aliphatic solvents (tetrachloroethylene -- PCE, and carbon tetrachloride -- CT) was studied in laboratory microcosms derived from hydrocarbon-contaminated groundwater aquifers. Some MAH, such as toluene and o-xylene, were completely degraded to CO <sub>2</sub> and CH <sub>4</sub> by mixed methanogenic cultures from a creosote-contaminated aquifer. This degradation was inhibited by the addition of accessory electron acceptors (oxygen, nitrate, sulfate), indicating acclimation of the microbial community to methanogenic conditions. The addition of preferred substrates, such as acetate, propionate, methanol, fatty acids, glucose, casamino acids, pepton, yeast extract, or acetone also inhibited MAH degradation, indicating that the presence of natural organic substrates may preclude anaerobic biodegradation of MAH in situ. Cyclohexane, CT, and high concentrations of toluene and o-xylene had a toxic effect. Under sulfate-reducing conditions, several MAH -- toluene, all three xylene isomers, and						
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19. ABSTRACT -- Continued

benzene were mineralized to  $\text{CO}_2$  by microorganisms from a petroleum-contaminated, sulfidogenic aquifer. Whereas toluene and xylenes were sequentially degraded in a mixture, benzene was degraded only if alone, or slowly transformed in a mixture with toluene. This explains previously reported recalcitrance of benzene under anaerobic conditions. The addition of preferred substrates (lactate, glucose, or yeast extract) to the cultures temporarily inhibited the degradation of MAH.

Methanogenic microcosms from the creosote-contaminated aquifer reductively dechlorinated hexa-, penta-, tetra-, tri-, and di-chlorobenzene. Monochlorobenzene was not reductively dechlorinated, but slowly degraded to  $\text{CO}_2$  through an unknown pathway. The addition of toluene did not accelerate the reductive transformations, which were deriving electrons possibly from unidentified organics on aquifer solids, or  $\text{H}_2$  in the anaerobic atmosphere, or internal storage granules in microorganisms (assessed by microscopy). Likewise, CT and PCE were transformed by microorganisms from three different subsurface sources without the addition of exogenous electron donors. CT was reductively dechlorinated to chloroform, and PCE to trichloroethylene, trans-1,2-dichloroethylene, and traces of vinyl chloride. Abiotic dechlorination, possibly due to microbial cofactors or metabolites, was also occurring, especially with CT. Toluene seemed to serve as a possible electron donor for reductive dechlorination of PCE in microcosms derived from one subsurface source only. However, in the case of CT, toluene did not accelerate reductive dechlorination. On the contrary, the dechlorination product from CT, chloroform, inhibited degradation of toluene.

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**ANAEROBIC MICROBIAL TRANSFORMATION OF AROMATIC  
HYDROCARBONS AND MIXTURES OF AROMATIC HYDROCARBONS  
AND HALOGENATED SOLVENTS**

Civil Engineering Report CE 319

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## ABSTRACT

Anaerobic transformation of mononuclear aromatic hydrocarbons (MAH), chlorobenzenes (CB), and mixtures of MAH and chlorinated aliphatic solvents was studied under anaerobic conditions, in laboratory microcosms containing five different environmental inocula. These inocula included aquifer solids from four contaminated groundwater aquifers and stable methanogenic consortia originally derived from anaerobic municipal sludge. Some of the substituted MAH (toluene, *o*-xylene) were completely degraded to CO<sub>2</sub> and CH<sub>4</sub> under methanogenic conditions by microflora from a creosote-contaminated aquifer, whereas toluene and all three isomers of xylene, as well as an unsubstituted MAH (benzene) were mineralized under sulfate-reducing conditions, by microorganisms from a gasoline-contaminated, sulfidogenic aquifer. Whereas the substituted MAH were degraded in mixtures with other MAH, benzene was mineralized only when used as sole substrate, or slowly degraded in a binary mixture with toluene. The addition of preferential microbial substrates (acetate, methanol, propionate, fatty acids, glucose, casamino acids, yeast extract, cysteine-HCl, or acetone for methanogenic cultures; lactate, yeast extract, or glucose for sulfate-reducing cultures) temporarily inhibited the degradation of MAH. The addition of accessory electron acceptors (O<sub>2</sub>, NO<sub>3</sub><sup>-</sup>, SO<sub>4</sub><sup>2-</sup>) to methanogenic cultures inhibited the degradation process. While the degradation of substituted MAH seems to be quite common in contaminated aquifers, unsubstituted MAH may be frequently recalcitrant, especially in mixtures with substituted MAH (which is usually the case).

The methanogenic microcosms derived from the creosote-contaminated aquifer reductively dechlorinated hexachlorobenzene (HCB), pentachlorobenzene (PeCB), 1,2,3,5-tetrachlorobenzene (TTCB), and 1,2,3-trichlorobenzene (TCB) to less chlorinated benzenes. 1,3-Dichlorobenzene (DCB) was a major product from 1,2,3-TCB, whereas 1,2,3-TCB was the main product from reductive dechlorination of other CBs; however, traces of monochlorobenzene (MCB) were also found in all the microcosms. These transformations occurred without the addition of potential electron donors, presumably by using unidentified donors on the aquifer solids, or H<sub>2</sub> from the anaerobic atmosphere, or by utilizing the internal microbial storage granules (poly-beta-hydroxybutyrate) which were identified in the cells. HCB, PeCB, and to a lower degree TTCB and TCB were also partially abiotically transformed, but at rates one to two orders of magnitude lower than in the active microcosms. The addition of yeast extract and pepton as electron sources for the active microorganisms accelerated the

reductive dechlorination in most cases, except for the conversion of PeCB to 1,2,3,5-TTCB. The addition of acetate slowed down the dechlorination rates with all the CB tested, except for the first step of PeCB transformation (reductive dechlorination to TTCB). Toluene, *p*-cresol, phenol, or glucose had no effect; however, there was a reverse effect of CB on toluene -- an inhibition of methanogenic degradation of this MAH in the microcosms that were otherwise readily degrading toluene. The least chlorinated CB, MCB, was not reductively dechlorinated but transformed through an unknown route and partially mineralized to CO<sub>2</sub>. The presence of benzoate inhibited the transformation of MCB, whereas acetate supported MCB degradation.

Numerous factors that can potentially influence the anaerobic transformation of chlorinated and nonchlorinated aromatic hydrocarbons merit consideration. 1) High initial concentrations of these compounds may be toxic or inhibitory to the microflora, especially if the microorganisms have not been exposed to these compounds before. Slow acclimation of the microflora to lower concentrations of these compounds, that occur at the outskirts of contamination plumes, gradually alleviates this effect, and the bacteria become tolerant to increasing concentrations of the pollutants. 2) Microorganisms are heterogeneously distributed, both in a spatial and a temporal sense, and it can not be expected that the transformations occur continuously in all the contaminated parts of an aquifer. 3) If the microflora is acclimated to the conditions of methanogenic fermentation, the addition of exogenous electron acceptors such as nitrate or sulfate may impair the capability of the microorganisms to degrade these compounds, although anaerobic respiration -- in theory -- is more favorable than fermentation. 4) pH will strongly influence the rates and extent of transformations, and there are different pH optima for different microbial communities. 5) The inorganic chemistry of the site is very important for successful biotransformation: higher concentrations of microbial nutrients will support more growth and therefore more pollutant degradation; furthermore, some of the products of microbial activity (e.g., free sulfide) may be toxic to microorganisms unless they are bound by appropriate inorganic species. 6) The presence of other organic compounds, either natural substrates or other pollutants, may either accelerate or slow down the transformation of other compounds, depending on the components of the mixture; in some cases there is no influence. Complex processes, such as preferential substrate utilization, sequential degradation, electron transfer between potential electron donors and acceptors, enzyme inhibition, or toxicity may be involved. 7) Aromatic hydrocarbons do not support, or support very infrequently the reductive dechlorination of CB,

whereas CB seem to inhibit anaerobic degradation of MAH; the addition of appropriate electron donors for dechlorination of CB may help in such cases.

Transformation of carbon tetrachloride (CT) and tetrachloroethylene (PCE) was studied under methanogenic conditions, in the presence or absence of several aromatic compounds (toluene, ethylbenzene, phenol, and benzoate). Microbial inocula for the experiments were derived from three ground water aquifers, contaminated by jet fuel or creosote. The results indicated that CT and PCE were reductively dechlorinated in all the examined cases (CT to chloroform [CF], and PCE to trichloroethylene [TCE], *trans*-1,2-dichloroethylene [DCE], and vinyl chloride [VC]). The electron donors used for the reductive transformation were the unidentified organic compounds present on aquifer solids, and/or H<sub>2</sub> from the anaerobic headspace. The addition of acetate and complex organic compounds, such as yeast extract and peptone, increased the reductive dechlorination rates. The addition of benzoate caused a decrease in rates of dechlorination, possibly because benzoate (which is initially reductively transformed under anaerobic conditions) was competing for the same sources of electrons. Phenol and ethylbenzene were not degraded, and their presence did not influence the transformation of CT or PCE. The results with toluene were mixed. This aromatic hydrocarbon was anaerobically degraded (to CO<sub>2</sub> and CH<sub>4</sub>) in some of the microcosms, and in a stable methanogenic consortium derived from the microcosms. CF, a product of CT dechlorination, inhibited the toluene degradation, whereas TCE, DCE, and VC did not. In most of the studied cases, the presence of toluene had no influence on reductive dechlorination of either CT, or PCE. Only in one case (microcosms derived from a JP-4 jet fuel contaminated aquifer), a connection could possibly be established between anaerobic degradation of toluene and simultaneous reductive dechlorination of PCE, suggesting that toluene might be used as an electron donor for reductive transformation of chlorinated solvents. This phenomenon could be very important in contaminated ground water aquifers *in situ*; however, its occurrence would depend on the distribution of microorganisms within an aquifer, microbial community structure, and various environmental conditions.

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## 1. INTRODUCTION AND STATEMENT OF WORK

Numerous toxic or carcinogenic aromatic and aliphatic compounds that are found as subsurface pollutants have become an increasingly serious problem nationwide; the development of successful approaches for their cleanup has become a necessity. The most common pollutants of concern include various monoaromatic hydrocarbons (MAH), polyaromatic hydrocarbons (PAH), nitrogen and sulfur heterocycles (NS), chlorinated benzenes (CB), and halogenated aliphatic solvents. The most frequent contamination sources are petroleum spills and leaks, landfill leachate intrusion, creosote leakage from unprotected impoundments, solvent leaks from underground storage tanks, energy and defense industry wastes as well as other industrial waste streams, and agricultural use of pesticides. Some of these contaminants can be partially removed by physical procedures; however, these methods become unproductive as the concentration of the contaminants decreases, and they also create a problem of contamination of another environmental compartment. A better approach is *in-situ* or on-site bioreclamation. So far, aerobic microorganisms have been employed for treatment of contaminated sites, and have proven to be quite successful in some cases, such as gasoline contamination (Raymond, 1974; Raymond et al., 1975; Lee et al., 1988) or vinyl chloride (VC)/dichloroethylene (DCE)/trichloroethylene (TCE) contamination (Semprini et al., 1992). The utilization of microbial processes under anoxic conditions, with the addition of substitute electron acceptors such as nitrate, has been rare (Hutchins et al., 1991). And yet, anaerobic conditions are to be expected in groundwater aquifers with significant concentrations of organic pollutants, because indigenous microorganisms rapidly utilize the available oxygen; unfortunately, it is not always feasible to reintroduce sufficient oxygen into contaminated sites. Furthermore, not all of the pollutants are amenable to aerobic transformation. Whereas MAH, PAH, NS, and aliphatic solvents with a low degree of halogenation can be completely degraded under aerobic conditions, highly halogenated aromatics and aliphatics are recalcitrant to oxidative transformation.

In the oxygen-devoid habitats, microbial respiration can occur in the presence of alternative exogenous electron acceptors, such as nitrate, ferric iron, or sulfate; when no exogenous electron acceptors are available, fermentation of some substrates can occur. Strictly anaerobic processes (sulfate reduction, fermentation/

methanogenesis) are of most interest because they can still function at low oxidation-reduction potentials (usually below -300 mV), when "easier" (more energy-yielding) acceptors, such as oxygen or nitrate, have been used up. From the standpoint of *in situ* bioreclamation, such processes are especially attractive because they create comparably little biomass, while having the potential to convert significant quantities of pollutants. To date, relatively little research on fermentative or sulfate-reducing transformation of MAH, PAH, or NS has been performed. Ward et al. (1980) observed slow anaerobic transformation of toluene in oil-polluted salt-marsh sediments. Wilson et al. (1986, 1987) reported slow disappearance of benzene, toluene, and xylenes in laboratory batch systems containing saturated aquifer solids from a petroleum-contaminated site. Grbić-Galić and Vogel (1987), working with methanogenic consortia originally derived from anaerobic sludge, observed complete conversion of  $^{14}\text{C}$ -labeled toluene and benzene to  $\text{CO}_2$  and  $\text{CH}_4$ . Numerous aromatic and aliphatic intermediates of transformation were detected. By using  $^{18}\text{O}$ -labeled water and GC/MS techniques, Vogel and Grbić-Galić (1986) showed that the initial transformation reaction was oxidative, yielding *p*-cresol from toluene and phenol from benzene, respectively; the oxygen was derived from water. Haag et al. (1991) showed some indications that toluene and *p*-xylene were transformed in subsurface samples with sulfate as a terminal electron acceptor. Non-oxygenated nitrogen heterocyclic compounds were also recently shown to undergo an initially oxidative (hydroxylation) transformation reaction under fermentative/methanogenic conditions (Pereira et al., 1988). The nitrogen heterocyclic compounds that are completely degraded to  $\text{CO}_2$  and  $\text{CH}_4$  under methanogenic conditions include indole (Madsen et al., 1988; Godsy et al., 1989 a), quinoline, and isoquinoline (Godsy et al., 1989 a). Bak and Widdel (1986) reported degradation of pyridine, indole, and quinoline by a marine isolate (*Desulfobacterium indolicum*) under sulfate-reducing conditions. Kuhn and Suflita (1989) observed slow transformation of pyridine and picoline isomers in saturated subsurface microcosms containing sulfate as an electron acceptor. So far, only one report has been published on complete degradation of a sulfur heterocycle (benzothiophene) under anaerobic (methanogenic) conditions (Godsy and Grbić-Galić, 1989 b).

Chlorinated benzenes, especially the highly chlorinated ones, should be susceptible to initially reductive, rather than oxidative, transformation. This was indeed shown by Tiedje et al. (1987), Bosma et al. (1988), and Fathepure et al. (1988). Hexa- to trichlorobenzenes can be reductively dechlorinated to dichlorobenzenes or all the

way to monochlorobenzene, which is a compound susceptible to aerobic biodegradation (Bosma et al., 1988). Reductive dechlorination is a process where CB act as electron acceptors or sinks; the electrons for the reduction are derived from various organic substrates. So far, however, it has not been shown that microorganisms can obtain energy by utilizing CB as respiratory electron acceptors, although such evidence had been obtained for other chlorinated aromatics, e.g. chlorobenzoate (Dolfing and Tiedje, 1986)..

Similar to chlorobenzenes, chlorinated aliphatic solvents such as carbon tetrachloride (CT) or tetrachloroethylene (PCE) are susceptible to reductive transformation (Vogel et al., 1987). PCE seems to be completely recalcitrant under aerobic conditions (Bouwer et al., 1981) because it is too oxidized and thus a poor electron donor. On the contrary, both PCE and CT will readily accept electrons from suitable electron donors and be quickly reductively dechlorinated by microorganisms under anaerobic conditions, including the anaerobic subsurface microflora (Bouwer and McCarty, 1983; Parsons et al., 1984, 1985; Vogel and McCarty, 1985). The dechlorination products that are sequentially formed are TCE, DCE isomers, and VC (Vogel and McCarty, 1985; Fathepure et al., 1987; Fathepure and Boyd, 1988; Bagley and Gossett, 1990). Sometimes, under methanogenic conditions, ethylene (Freedman and Gossett, 1989) and CO<sub>2</sub> (Bouwer and McCarty, 1983; Vogel and McCarty, 1985) are also formed. The reductive dechlorination process can be mediated by microbial electron carriers such as NAD, flavins, cytochromes, vitamin B<sub>12</sub>, glutathione, or others, which transfer electrons from an electron donor to the chlorinated compound (Wade and Castro, 1973; Kiečka and Gonsior, 1984; Vogel et al., 1987; Gantzer and Wackett, 1991). The products of reductive dechlorination (lesser chlorinated aliphatic compounds) are sometimes more toxic than the parent compounds (e.g., CF from CT, and VC from PCE). They are also more persistent under anoxic conditions, i.e., transformed at progressively slower rates as the degree of chlorination decreases (Vogel et al., 1987).

It is conceivable that chlorinated solvents and nonchlorinated aromatic hydrocarbons might be found as pollutant mixtures in numerous ground water aquifers. Examples are the Picatinny Arsenal site, New Jersey (Wilson et al., 1989), and the Traverse City site, Michigan (Sewell and Gibson, 1991). Since chlorinated solvents can act as electron acceptors, and aromatic hydrocarbons as electron donors under anaerobic conditions, theoretically it might be possible that microbially

catalyzed transformation reactions occurred that would couple the oxidation of petroleum hydrocarbons to reduction of chlorinated solvents. These processes, if occurring *in situ*, could be very important in controlling the fate and transport of both categories of pollutants. Furthermore, they would be of a great significance for bioreclamation of the contaminated sites. Wilson et al. (1989) have observed simultaneous removal of TCA, TCE, and alkylbenzenes (especially toluene) in anaerobic microcosms derived from the Picatinny Arsenal ground water aquifer. Sewell and Gibson (1991), using microcosms derived from the methanogenic zone of a ground water aquifer contaminated by JP-4 jet fuel and chlorinated ethenes (U.S. Coast Guard Air Station, Traverse City, MI), found that PCE transformation could possibly be linked to simultaneous toluene oxidation. TCE, DCE, acetate, and methane were the products found in this mixture.

The first objective of this project was to study anaerobic microbial transformation of MAH, PAH, and NS model compounds under strictly anaerobic (sulfate-reducing and fermentative/ methanogenic) conditions, by subsurface microorganisms obtained from different contaminated groundwater sites. For comparison, stable methanogenic consortia derived from sewage sludge by enrichment on ferulate or toluene as sole substrates were also used as inocula. The research was planned at three levels: 1) batch "microcosms" containing saturated subsurface solids, spiked with compounds of interest and incubated under the conditions which attempted to simulate natural conditions; 2) suspended mixed cultures enriched from the microcosms; 3) pure cultures isolated from mixed cultures. Transformation intermediates, routes, rates, and the most important influencing factors (e.g., pH, temperature, agitation, presence of alternate electron donors) were to be examined. In addition to the work with single substrates, it was also proposed to study biotransformation processes in mixtures of various aromatic compounds, and to attempt electron acceptor perturbation experiments using oxygen, nitrate, and sulfate (where applicable) as perturbants. Among the compounds chosen for study, only MAH were biodegraded by the available inocula within a two-year incubation period. PAH and NS compounds were therefore abandoned, and only MAH were further studied.

The second objective was to examine the anaerobic degradation of CB model substrates alone, or in mixtures with MAH aromatics. We wanted to test a hypothesis that MAH could be used as electron donors/ carbon sources, and CB as electron acceptors by microorganisms in the absence of other microbial electron acceptors



The third objective was to study the interactions between MAH as potential electron donors, and chlorinated aliphatic solvents (PCE, CT) as potential electron acceptors in microbial reductive dechlorination of these compounds. Such processes, if indeed occurring, would be of a great significance for restoration of contaminated sites containing mixtures of hydrocarbons and chlorinated solvents.

## 2. MATERIALS AND METHODS

### CONSTRUCTION AND MAINTENANCE OF MICROCOSMS AND ENRICHMENTS

The microcosms and enrichment cultures were all prepared in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Michigan). The culture containers were 250-mL screw-cap bottles that had been scrupulously prewashed and sterilized before they were brought into the anaerobic chamber. The bottles were preincubated in the anaerobic chamber for at least a day to remove all traces of oxygen; after this period, 15-100 g of aquifer material (microcosms) or 30 mL of inoculum (enrichments), together with prereduced defined mineral medium to make up to 200 mL of total solids+liquid volume, were added to each bottle. The remaining volume in the bottles was anaerobic headspace (10% CO<sub>2</sub>, 5% H<sub>2</sub>, and 85% N<sub>2</sub>; or 30% CO<sub>2</sub> and 70% N<sub>2</sub> in CB experiments). The bottles were sealed with Mininert<sup>TM</sup> valves (Alltech Associates Inc., Deerfield, Illinois). The nonchlorinated aromatic substrates were spiked as stock solutions, to yield the final concentrations of 50  $\mu$ M to 12.5 mM for each compound. CB were spiked as stock solutions in methanol or toluene, to yield the final concentrations of 1-15  $\mu$ M. The concentration of methanol in CB-amended microcosms was about 0.25-0.50  $\mu$ M. Nonhydrocarbon electron donors used in some experiments of CB transformation were benzoate (5 mM), phenol (5-10 mM), ferulate (5 mM), and glucose (0.25 mM). The microcosms and enrichments were incubated in the glove box, in the dark, at 35°C (fermentative/ methanogenic) or 20°C (sulfate-reducing cultures). At least duplicate or triplicate microcosms or enrichments were used for each combination of substrates and conditions studied. In addition to the active cultures, duplicate sterile controls were used; the controls that contained aquifer solids were autoclaved for 20 minutes at 121°C on three consecutive days before the medium was added to the bottles. After the transformations had begun, regular  $\alpha$  spiking with aromatic substrates and sulfate was performed as necessary. The aromatic compounds in neat form were injected directly into active microcosms with a 10- $\mu$ L syringe. Sulfate (an anaerobic solution of MgSO<sub>4</sub>) was added to sulfate-reducing microcosms with a 3-mL syringe.

Primary enrichment cultures were prepared from microcosms by transferring solids (10 g) and liquid (20-30 mL) to autoclaved 250-mL bottles. These bottles were then filled with 170-180 mL of the prereduced defined medium and spiked with

appropriate substrates in concentrations up to 200  $\mu\text{M}$ . Further enrichments were prepared by transferring only the liquid portion of primary enrichments into fresh medium (10-30% inoculum). Enrichments that no longer contained aquifer solids were used for most suspended culture experiments. In CB tests where the influence of various electron donors on CB transformation was studied, the cells from the enrichments were harvested by centrifugation and washed with prereduced defined medium before inoculation into respective media. Isolation of pure cultures from fermentative/ methanogenic enrichments was attempted by using the agar-roll tube method (Hungate, 1969). The medium utilized in this procedure was the prereduced defined medium containing 15 g  $\text{L}^{-1}$  agar and the appropriate aromatic substrate.

### Substrates Tested


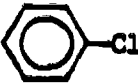
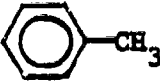
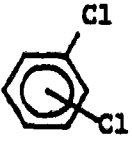
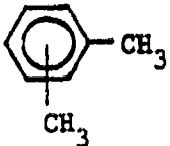

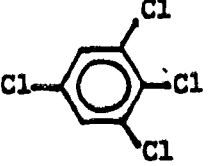
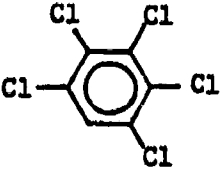
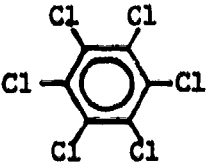
The aromatic hydrocarbon substrates tested for biotransformability included MAH and CB compounds. The chemicals and their structural formulae are summarized in Table 1.

The chlorinated aliphatic hydrocarbon substrates included PCE and CT.

### Chemicals

Most of the chemicals used were purchased from Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI) and were greater than 99.9% pure. 1,2,4-Trichlorobenzene (1,2,4-TCB) and 1,2,4,5-tetrachlorobenzene (1,2,4,5-TTCB) were obtained from Chem Service (West Chester, PA). Monochlorobenzene (MCB) and 1,2-dichlorobenzene (1,2-DCB) were purchased from Fluka AG Buchs SG (Switzerland). PCE, CT, and sodium acetate were purchased from Baker Chemical Co. (Phillipsburg, NJ). Acetic acid was obtained from Mallinkrodt, Inc. (Paris, KY). Benzoic acid was a product of Baker and Adamson Co. (New York, NY). Gases (helium, zero grade; hydrogen, zero grade; 95% argon : 5% methane mixture; and compressed air) were purchased from Liquid Carbonic (San Carlos, CA). [*Methyl*- $^{14}\text{C}$ ]toluene, [ring- $^{14}\text{C}$ ]toluene, [*methyl*- $^{14}\text{C}$ ]o-xylene, [ring- $^{14}\text{C}$ ]MCB, and [ring- $^{14}\text{C}$ ]1,4-DCB were purchased from Sigma. Liquid scintillation cocktail (Universol-Biodegradable-Nontoxic-Nonflammable) was obtained from ICN Biomedicals, Inc. (Irvine, CA).

Table 1. Aromatic hydrocarbon substrates tested for anaerobic biotransformability

Compound	Chemical Structure	Compound	Chemical Structure
<b>Monoaromatic Hydrocarbons</b>		<b>Chlorinated Benzenes</b>	
Benzene		Monochlorobenzene	
Toluene		Dichlorobenzene Isomers	
Xylene Isomers		Trichlorobenzene Isomers	
		Tetrachlorobenzene Isomers	
		Pentachlorobenzene	
		Hexachlorobenzene	

## Growth Medium

The composition of the defined prereduced mineral growth medium was a modification of several previous recipes (Owen et al., 1979; Godsy, 1980; Shelton and Tiedje, 1984; Ridgway et al., 1989; Tong et al., 1990). The composition of the medium is shown in Table 2. The final pH of the medium was adjusted to 6 or 7, depending on the requirements of the particular microbial communities. The medium was autoclaved (excluding  $\text{NaHCO}_3$ ,  $\text{FeS}$ , and vitamins) at  $121^\circ\text{C}$  for 20 minutes, then purged with  $\text{N}_2/\text{CO}_2$ , sealed, and brought into the anaerobic chamber. Vitamins,  $\text{FeS}$ , and  $\text{NaHCO}_3$  were added to the medium from sterile, anaerobic stock solutions. In some cases (specified in Results and Discussion section), vitamins were not added to the medium. In some of the experiments with chlorinated aliphatic solvents, organic compounds other than the solvents and/or aromatic hydrocarbons were added to the medium. "Carbon-poor" medium (CP) received the addition of  $30 \text{ mg L}^{-1}$  ( $0.33 \text{ mM}$ ) sodium acetate. "Carbon-rich" medium (CR) received the additions of  $2 \text{ g L}^{-1}$  ( $25 \text{ mM}$ ) sodium acetate,  $1 \text{ g L}^{-1}$  yeast extract, and  $0.1 \text{ g L}^{-1}$  Bacto-peptone (Difco, Detroit, MI). Chlorinated hydrocarbons and nonchlorinated aromatic hydrocarbons were added directly to the medium after autoclaving. Phenol and benzoate were added as concentrated stock solutions in water.

## Inocula

Aseptically sampled, anaerobically handled aquifer solids from several different sites were used as sources of microbial inocula. The first site was a JP-4 fuel contaminated aquifer at Tyndall Airforce Base (FL). The samples were provided by Department of the Air Force (Headquarters Air Force Engineering and Services Center, Tyndall Air Force Base; courtesy of Capt. C.M. Vogel). Drilling was performed with a hollow-stem auger, and the samples were obtained from split-spoon samplers, using sterilized metal cylinders (Capt. C. M. Vogel, personal communication). The solids were contaminated by 10 to 20 g total hydrocarbons/kg soil. The samples were stored at  $4^\circ\text{C}$  during shipping. Before the onset of the experiments, the samples were preincubated in prereduced defined medium without the addition of any substrates, in an anaerobic glove-box at  $35^\circ\text{C}$  for an hour. The liquid phase was then withdrawn with a syringe, and replaced with fresh medium, in order to reduce the organic load on the solids.

Table 2. Composition of the prereduced defined mineral medium

Compound	Concentration (mg/l)	Concentration (mM)
$\text{KH}_2\text{PO}_4$	272	2.0
$\text{K}_2\text{HPO}_4$	348	2.0
$\text{NH}_4\text{Cl}$	535	10.0
$\text{MgSO}_4 \times 7\text{H}_2\text{O}$	125	0.51
$\text{CaCl}_2 \times 2\text{H}_2\text{O}$	70	0.48
$\text{FeCl}_2 \times 4\text{H}_2\text{O}$	20	0.1
<b>Trace Minerals:</b>		
$\text{H}_3\text{BO}_3$	0.6	$9.7 \times 10^{-3}$
$\text{ZnCl}_2$	0.2	$1.5 \times 10^{-3}$
$\text{Na}_2\text{MoO}_4 \times 2\text{H}_2\text{O}$	0.2	$0.8 \times 10^{-3}$
$\text{NiCl}_2 \times 6\text{H}_2\text{O}$	1.5	$6.3 \times 10^{-3}$
$\text{MnCl}_2 \times 4\text{H}_2\text{O}$	2.0	$10.1 \times 10^{-3}$
$\text{CuCl}_2 \times 2\text{H}_2\text{O}$	0.2	$1.2 \times 10^{-3}$
$\text{CoCl}_2 \times 6\text{H}_2\text{O}$	3.0	$12.6 \times 10^{-3}$
$\text{Na}_2\text{SeO}_3$	0.04	$0.2 \times 10^{-3}$
$\text{Al}_2(\text{SO}_4)_3 \times 18\text{H}_2\text{O}$	0.2	$0.3 \times 10^{-3}$
<b>Buffer:</b>		
$\text{NaHCO}_3$	1200	14.3
<b>Resazurin</b>	1.0	0.004
<b>Reducing Agent:</b>		
$\text{FeS}$ (amorphous*)	54.8	0.62
<b>Vitamins:</b>		
Biotin	0.002	
Folic ac id	0.002	
Pyridoxine hydrochloride	0.02	
Riboflavin	0.005	
Thiamine	0.005	
Nicotinic ac id	0.005	
Pantothenic acid	0.005	
<i>p</i> -Aminobenzoic ac id (PABA)	0.005	
Cyanocobalamin ( $\text{B}_{12}$ )	0.005	
Thioctic (lipoic) acid	0.005	
Coenzyme M (Mercapto- ethanesulfonic acid)	0.1	

\* Brock and O'Dea (1977)

The second site was an aviation gasoline-contaminated aquifer at the U.S. Coastguard Air Station at Traverse City (MI). Core samples of coarse-grained aquifer material from this site were provided by the U.S. Environmental Protection Agency (Ada, OK; courtesy of S. Hutchins). The samples were collected aseptically with an auger under anaerobic conditions in August, 1989. The core sample used in this study was taken from below the water table, at 77 to 80 m below ground surface, from an actively methanogenic zone. It contained very low concentrations of nitrogen and phosphorus ( $\text{mg L}^{-1}$  quantities), and  $9.5 \text{ mg L}^{-1}$  sulfate (S. Hutchins, personal communication). The sample was stored at  $4^{\circ}\text{C}$  in a sealed Mason jar until use. Before the onset of the experiment, it was preincubated in prereduced defined medium without the addition of any substrates, at room temperature ( $20\text{--}25^{\circ}\text{C}$ ) in an anaerobic chamber for 24 days.

Aquifer solids from Pensacola (FL) aquifer were provided by E.M. Godsy (U.S. Geological Survey, Menlo Park, CA). The Pensacola aquifer consists of fine-to-coarse sand deposits, interrupted by discontinuous silts and clay. The upper 30 m of the aquifer are contaminated by creosote and pentachlorophenol. The samples were obtained from an actively methanogenic, sandy zone of the aquifer, downgradient from the contamination source, from the depth of approximately 6 m. The ground water at this depth contained tens of  $\text{mg L}^{-1}$  of nitrogen heterocycles, simple PAH, and phenols (Goerlitz et al., 1985). The indigenous microorganisms had been shown to anaerobically degrade aromatic and heterocyclic constituents of the water-soluble creosote fraction (Godsy et al., 1989; Godsy and Grbić-Galić, 1989). The aseptic sampling was performed with a hollow-stem auger and a split-spoon core sampling device. After the sampler was withdrawn from the borehole and split lengthwise, a portion of the core was removed with a sterile spatula. The center of the core was then subsampled by pushing a sterile brass tube into the core, extruded with a sterile syringe plunger, and stored (at  $4^{\circ}\text{C}$ ) in sterile sealed containers previously flushed with argon.

Samples of gasoline-contaminated sandy silt from the Seal Beach (CA) site were provided by Dr. Harry Ridgway (Orange County Water District, Fountain Valley, CA). They were collected with a steam-cleaned 0.76 m-diameter bucket auger at a depth of 0.25 m above the water table. After collection, the contents of the auger were transferred to a 20-L plastic bucket flushed with  $\text{N}_2$  and sealed with air-tight snap lids. The sediment was stored at  $4^{\circ}\text{C}$  until use.

In addition to the environmental samples described above, we also used stable methanogenic cultures originally enriched from anaerobic municipal sludge by using ferulic acid (an aromatic derivative of lignin degradation) as sole organic carbon source (Grbić-Galić and Young, 1985). The cultures had been maintained on ferulic acid for five years before use in these experiments. Ferulic acid consortia were tested for their capability to transform MCB, 1,4-DCB, and 1,3-DCB. Toluene consortia that were derived from the ferulate consortia by enrichment on toluene were used to study the biodegradability of ethylbenzene and xylenes. Naphthalene-degrading consortia, which were derived from the ferulate consortia in two steps (benzene enrichment and subsequently naphthalene enrichment; Grbić-Galić, 1989) were used to test biodegradability of acenaphthene.

## **ANALYTICAL METHODS**

### **Gas Chromatography (GC)**

The concentrations of MAH, naphthalene, and CB were measured by withdrawing 300  $\mu$ L of headspace from sample bottles with a 500- $\mu$ L gas-tight syringe and injecting the headspace into a Carlo Erba Fractovap 2900 Series Gas Chromatograph (GC, Carlo Erba Strumentazione, Milan, Italy) equipped with a photoionization detector (PID, HNU Systems Inc., Model PI-52-02; 10 eV lamp) and a 30m x 0.53 mm (ID) DB-624 megabore fused silica capillary column (3.0  $\mu$ m film thickness; J&W Scientific, Folsom, CA). The operating conditions were an injection port temperature of 240°C, a detector temperature of 250°C, helium carrier gas at a column head pressure of 0.7 kg/cm<sup>2</sup>, and helium make-up gas at a flow of 30 mL min<sup>-1</sup>. Analyses were isothermal (90°C for MAH and PAH; 125°C for MCB, DCB, and TCB; 165°C for higher chlorinated CB) and splitless (split closed for 30 sec). The data from GC were collected and processed with the Nelson Analytical Inc. 3000 Series Chromatography Data System. Standards for headspace analyses were prepared by spiking a methanolic stock solution of the aromatic compound into a Mininert-sealed bottle that contained 200 mL of water. The transfer of the stock solution was made with a gas-tight 500- $\mu$ L syringe. The amount of stock solution added to the standard bottle was determined gravimetrically by weighing the syringe immediately before and after spiking. The aqueous concentration of aromatic compounds in standards was estimated by using Henry's Law constants obtained from



literature (Mackay and Shiu, 1981), and by using the equation derived from the definition of Henry's Law. The aqueous concentration of aromatic compounds in microcosms and enrichments was determined by comparison of peak areas to standards. It must be stressed that the mass of hydrocarbon sorbed to solids in microcosms was not represented in this measurement, which resulted in an underestimation of the calculated amount of aromatic substrate when the substrate was completely consumed. This problem was not present in suspended enrichments.

CT, CF, DM, and CM were also assayed by headspace analysis, using a Tracor gas chromatograph (Tracor Instruments, Austin, TX) with a linearized Ni-63 electron-capture detector, equipped with a packed column (10% squalene on Chromosorb W/AW). The GC was connected to a Nelson Systems interface (Perkin Elmer/Nelson Systems Inc., Cupertino, CA). Argon/methane mixture (95% : 5%; Liquid Carbonic, San Carlos, CA) was utilized as carrier gas, at a rate of 7.75 mL min<sup>-1</sup>. The same mixture was used as make-up gas, at a rate of 70 mL min<sup>-1</sup>. Half-mL gaseous samples were analyzed isothermally at 70°C. The injector temperature was 135°C, and the detector temperature 258°C.

VC was analyzed on a HP 5890 gas chromatograph (Hewlett-Packard Co., Avondale, PA) with a Model 4420 electronic conductivity detector. The column was DB-624 capillary column, 30 m x 0.553 mm, with 3.0 µm film thickness (L&W Scientific, Folsom, CA). Helium was used as carrier gas. The column pressure was 0.5 kpa. Hydrogen was used as make-up gas, at a rate of 20 mL min<sup>-1</sup>. Headspace analyses (0.5-mL samples) were performed isothermally, at 35°C. The detector temperature was 800°C, and the injector temperature 210°C.

Methane was analyzed by isothermal GC (120°C), by injecting 0.5 mL of headspace into a Hewlett-Packard 5730A GC (Hewlett-Packard, Avondale, PA) with a flame-ionization detector and a 60/80 Carbosieve column (1.7 m x 3 mm; Supelco, Inc., Bellafonte, PA).

Gaseous samples for the GC analyses of chlorinated aliphatics and methane were withdrawn and injected by a 0.5-mL pressure-lock series A-2 syringe (Alltech Applied Science, San Jose, CA).

## Gas Chromatography/ Mass Spectrometry (GC/MS) Analyses

GC/MS was used for tracing NS compounds, and for analyzing potential transformation intermediates in CB samples. For NS analysis, a polar DB-WAX column (30 m x 0.25 mm i.d.) with a bonded carbowax phase (0.25  $\mu$ m; J&W Scientific, Inc., Folsom, CA) was used; it was connected directly to the ion source of the mass spectrometer which was operated in the electron impact mode. Samples (2 mL of the culture fluid) were acidified to pH 1.5 and extracted with 1 mL of diethyl ether. The ether extract was injected splitless onto the column. The oven temperature was programmed from 50°C at 10°C min<sup>-1</sup>, to the maximum temperature limit of the column (250°C). For the analysis of metabolites produced from MAH, 2.75 mL of the culture fluid was acidified and extracted with 1 mL of diethyl ether. For CB metabolites, the same volume of the culture fluid was extracted with 1 mL of hexane, without acidification. The samples were not derivatized. Analyses were performed on a HP Model 5890 GC with a 60-m x 0.32 mm (ID) DB-5 fused silica capillary column with 1  $\mu$ m film thickness (J&W Scientific), coupled with a 5970 Series Mass Selective Detector (Hewlett-Packard Company, Palo Alto, CA). The operating conditions were an injection port temperature of 275°C, transfer line temperature of 280°C, and helium carrier gas at a flow of 30 mL min<sup>-1</sup>. For MAH metabolites, the temperature program was as follows: 40°C -- hold 5 min; to 120°C (at a rate of 2°C min<sup>-1</sup>), and then to 200°C (at 4°C min<sup>-1</sup>). For CB metabolites, the program was as follows: initial temperature 60°C -- hold 2 min; increase to 280°C at a rate of 4°C min<sup>-1</sup>; hold at final temperature for 10 min. The peaks were identified by comparison of the spectra with authentic standards or by using the National Bureau of Standards Library spectra.

## High Performance Liquid Chromatography (HPLC)

Accessory aromatic substrates (benzoate, ferulate, phenol, *p*-cresol) were analyzed by HPLC. Samples were centrifuged at 4800 rpm for 5 min to remove particulates. The HPLC system was a Perkin-Elmer Series 400 Liquid Chromatograph (Perkin-Elmer, Norwalk, CT) coupled with SP 4020 Data Interface ((Spectra-Physics, Germany). It was equipped with a C-18 reverse phase 250 mm x 4.6 mm (ID) column (Alltech Assoc., Deerfield, IL) and a HP 1050 Series Variable Wavelength Detector (Hewlett-Packard, Avondale, PA). Ferulate was monitored using a mixture of 60% of the 50 mM acetate buffer (pH 4.5) and 40% of methanol as eluant at a flow rate of 1 mL min<sup>-1</sup> (isocratic analysis); the detector wavelength was adjusted to 308 nm. Benzoate,

*p*-cresol, and phenol were monitored using 70% of the acetate buffer and 30% of methanol as eluant at a flow rate of 1 mL min<sup>-1</sup> (isocratic analysis); the detection wavelength was 280 nm.

### Sulfate and Sulfide Analyses

Sulfate was analyzed on a Dionex Series 4000i Ion Chromatograph (IC) using an electrochemical conductivity detector. The eluant was 0.75 mM sodium bicarbonate/ 2.2. mM sodium carbonate, at a rate of 2 mL min<sup>-1</sup>. The regenerant was 0.025 N sulfuric acid. The data from IC were collected and processed with the Nelson Analytical Inc. 3000 Series Chromatography Data System. The calibration of IC was performed with external standards.

Dissolved sulfide concentration (H<sub>2</sub>S, HS<sup>-</sup>, S<sup>2-</sup>) was determined using the spectrophotometric methylene blue method (Cline, 1969).

### <sup>14</sup>C Analysis

<sup>14</sup>C-activity in liquid and gaseous samples was determined on a Tricarb Model 4530 scintillation spectrometer (Packard Instrument Co., Downers Grove, IL). Counting efficiency corrections were made using the external standard channels ratio method (Bell and Hayes, 1958). Three separate 1-mL liquid samples were counted for each analysis (Grbić-Galić and Vogel, 1987). All three samples were mixed with 10 mL of liquid scintillation cocktail, but one was pretreated with 1 mL of 1N HCl, the other with 1 mL of 1N NaOH, whereas the third received no pretreatment. The pretreated samples were nitrogen-stripped before adding the scintillation cocktail. These measurements were used to determine the <sup>14</sup>C-activity in the volatile, nonvolatile, and CO<sub>2</sub> fractions, as described by Grbić-Galić and Vogel (1987).

### Microscopy

Microbial cell counts were determined in a 10-μL sample spread over a 1 cm<sup>2</sup> area on a microscope slide. The samples were heat-fixed and stained with acridine orange (0.01%) for two minutes, then washed with water. The cells were observed under oil immersion in an epifluorescence microscope (Olympus Optical Co., Tokyo, Japan). Sixteen to 20 fields per sample were counted and the average cell count per

field was used to calculate the total cell count, given that the area of the field was  $1.7 \times 10^{-3} \text{ mm}^2$ .

### **Protein and Poly-Beta-Hydroxybutyrate Assays**

The microcosm and culture fluids were analyzed for protein with the method after Bradford (1976) to follow microbial growth. Poly-b-hydroxybutyrate in microbial cells was visualized by staining with Sudan black and using bright field microscopy.

### 3. RESULTS AND DISCUSSION

#### METHANOGENIC DEGRADATION OF MAH

##### A. Initial Adaptation

Aquifer solids from Pensacola (FL) were used to set up microcosms that were initially fed mixtures of substituted MAH (toluene, ethylbenzene, *o*-xylene, and *p*-xylene), or of unsubstituted aromatic hydrocarbons (naphthalene and benzene). Low concentrations were used in both mixtures (0.12 mM total hydrocarbons). Half of the microcosms with substituted MAH received also the addition of 0.46 mM *p*-cresol, and half of those with unsubstituted hydrocarbons, phenol. *p*-Cresol had been shown previously to be an important intermediate of methanogenic toluene degradation, and phenol of benzene degradation, respectively (Vogel and Grbić-Galić, 1986); these compounds were added to see how they would influence the adaptation of the aquifer microflora to hydrocarbon degradation. During 250 days of incubation in an anaerobic chamber at 35°C, no transformation of either benzene or naphthalene occurred (Table 3). In the substituted MAH series, only one microcosm from each group exhibited transformation activity towards some of the MAH; autoclaved controls showed no activity whatsoever. In the microcosm without *p*-cresol, toluene transformation started after about 120-days lag period, and *o*-xylene degradation after 255 days. In the microcosm with *p*-cresol, the acclimation lag for toluene transformation was about 100 days, and that for *o*-xylene, 200 days; *p*-cresol itself was degraded in less than 30 days of incubation. It seems that *p*-cresol might have been supporting growth of biomass, which allowed for faster enrichment of hydrocarbon-transforming activity. Upon refeeding of the microcosms with 0.100 mM toluene and 0.46 mM *p*-cresol, the degradation of both compounds proceeded without a lag. Ethylbenzene and *p*-xylene were not transformed.

In an attempt to study the adaptation to toluene degradation under various environmental conditions, a different batch of Pensacola aquifer material, previously used in experiments testing the degradation of creosote constituents (E.M. Godsy, personal communication), was used to set up new microcosms. The following conditions were tested:

- 1) toluene (0.08 mM) alone, microcosms incubated statically as before;
- 2) toluene alone, with microcosms shaken vigorously once per day;
- 3) toluene with *p*-cresol

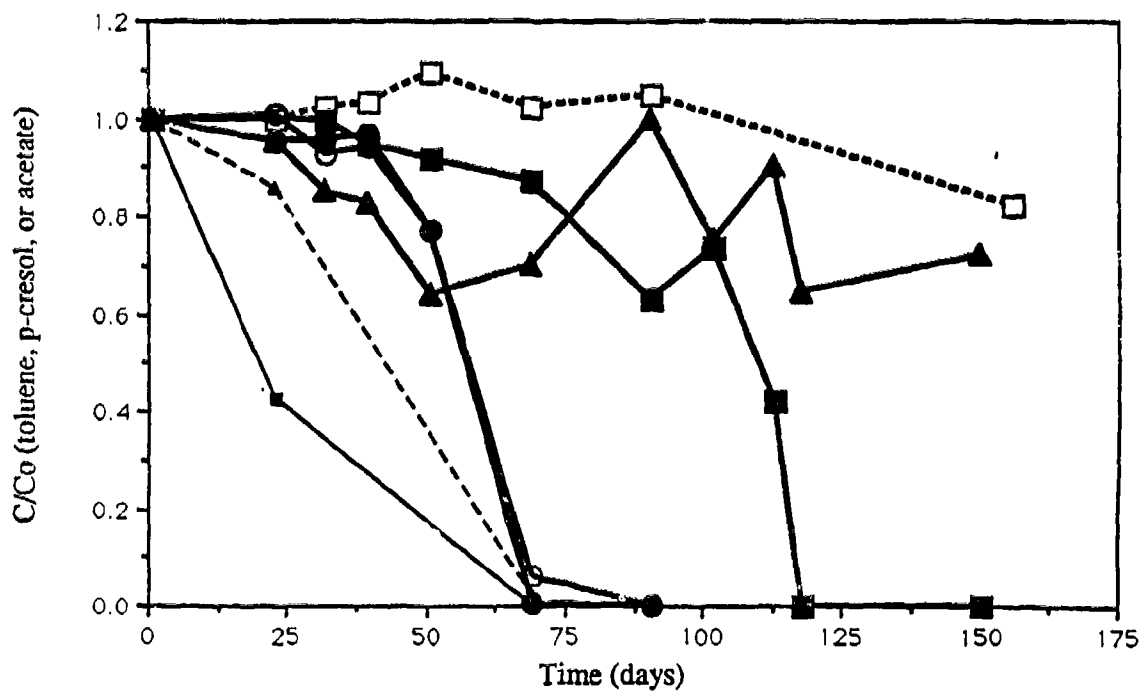
Table 3. Initial adaptation lags to toluene and *o*-xylene in Pensacola microcosms

	SUBSTRATE MIXTURE	INITIAL CONCENTRATION	DAYS UNTIL DEGRADED
Group 1	<i>p</i> -Cresol	460 $\mu$ M	80 days
	Toluene	40 $\mu$ M	100 days
	<i>o</i> -Xylene	40 $\mu$ M	200 days
	Ethylbenzene	40 $\mu$ M	not degraded
	<i>p</i> -Xylene	40 $\mu$ M	not degraded
Group 2	Toluene	40 $\mu$ M	120 days
	<i>o</i> -Xylene	40 $\mu$ M	255 days
	Ethylbenzene	40 $\mu$ M	not degraded
	<i>p</i> -Xylene	40 $\mu$ M	not degraded
Group 3	Toluene	320 $\mu$ M	not degraded
	<i>o</i> -Xylene	320 $\mu$ M	not degraded
	Ethylbenzene	320 $\mu$ M	not degraded
	<i>p</i> -Xylene	320 $\mu$ M	not degraded
Group 4	Phenol	570 $\mu$ M	80 days
	Benzene	150 $\mu$ M	not degraded
	Naphthalene	30 $\mu$ M	not degraded

(0.75 mM); 4) toluene with acetate (25 mM). The results are presented in Figure 1. Each data point in Figure 1 represents the average of 4 replicate microcosms. The sediment used in this experiment was mixed as much as possible in a large beaker inside an anaerobic glove box before distribution into bottles. As a result of this mixing, replicates in this experiment were reproducible, with a maximum coefficient of variation of 20 percent. The lag time before the onset of degradation was about 50 days for both the static and agitated microcosms. The lag time using this sediment was considerably shorter than the lag times observed with samples of Pensacola sediment that had not been recently exposed to creosote compounds (Table 3). In the microcosms to which *p*-cresol was added, the lag time was increased to about 100 days; in the microcosms to which acetate was added, the lag time was increased to more than 175 days. Degradation of both *p*-cresol and acetate began without a lag. These findings suggest that *p*-cresol degrading microorganisms and aceticlastic methanogens are not the rate-limiting populations in the complex sequential toluene degradation process.

## B. Primary Enrichments

After the third refeeding, active microcosms were used as the source for enrichment of suspended microbial consortia degrading toluene and *o*-xylene. This was accomplished in two steps. In the first step, 10 g of aquifer solids and 20 mL of the culture fluid from the microcosms were transferred into 180 mL of the prereduced defined medium, and amended with 5 mg L<sup>-1</sup> of toluene and/or *o*-xylene. Transformation activity was detected after 1-2 weeks of incubation. Upon refeeding with the aromatic hydrocarbons, the degradation resumed and was completed in 2-3 weeks. In the second step of enrichment, only the liquid portion of the primary enrichments (30 mL) was transferred into 170 mL of medium. The degradation activity resumed. Figure 2 shows the <sup>14</sup>C-label distribution in suspended cultures degrading [<sup>14</sup>C]toluene. After several refeedings, the consortia stabilized and increased the degradation rate (complete degradation of toluene and *o*-xylene to stoichiometric amounts of CO<sub>2</sub> and CH<sub>4</sub> in 1-2 weeks). The amount of methane formed during the degradation of toluene and *o*-xylene was consistently greater than 90% of the theoretically predicted 4.3 moles of methane per mole of toluene and 4.7 moles of methane per mole of *o*-xylene (Figure 3). In the consortia fed a mixture of both toluene and *o*-xylene, both compounds were simultaneously degraded.



Symbols:

- toluene concentration in killed biological control microcosms;
- toluene in microcosms incubated statically;
- toluene in microcosms incubated shaken;
- toluene and —■— *p*-cresol in microcosms amended with both toluene and *p*-cresol;
- ▲— toluene and —▲— acetate in microcosms amended with both toluene and acetate.

Figure 1. Effect of various treatments on the length of the adaptation lag before the onset of toluene degradation



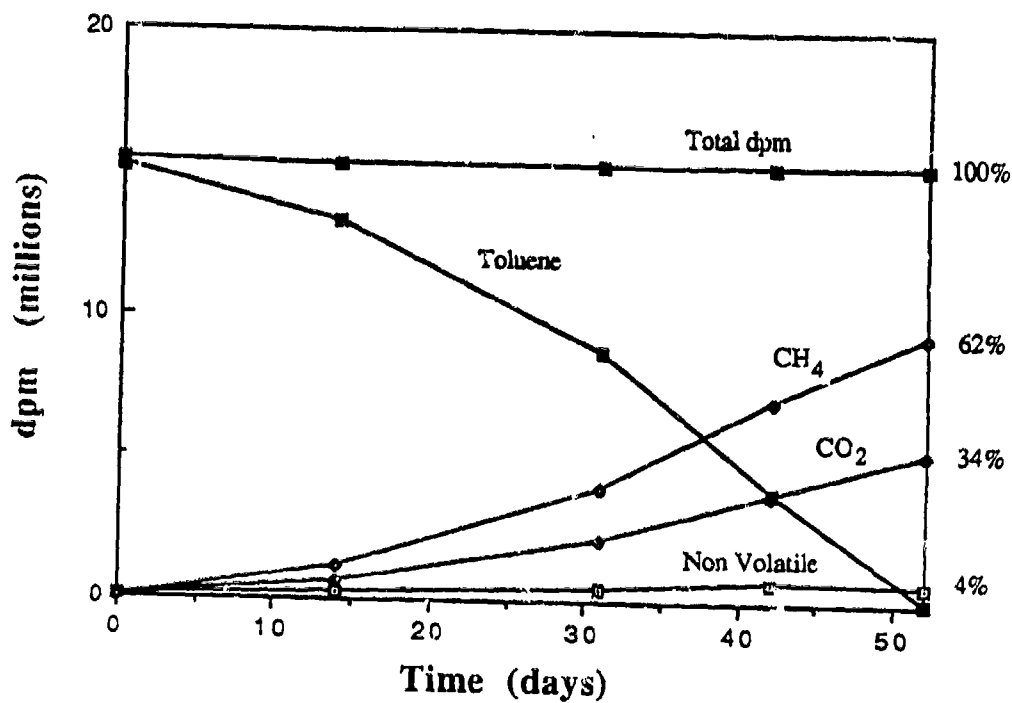


Figure 2.  $^{14}\text{C}$ -label distribution in mixed methanogenic cultures fed  $^{14}\text{C}$ -toluene. The nonvolatile fraction contains degradation intermediates and cell-bound  $^{14}\text{C}$ . Both  $^{14}\text{C}$ -ring- and  $^{14}\text{C}$ -methyl-toluene yielded identical results

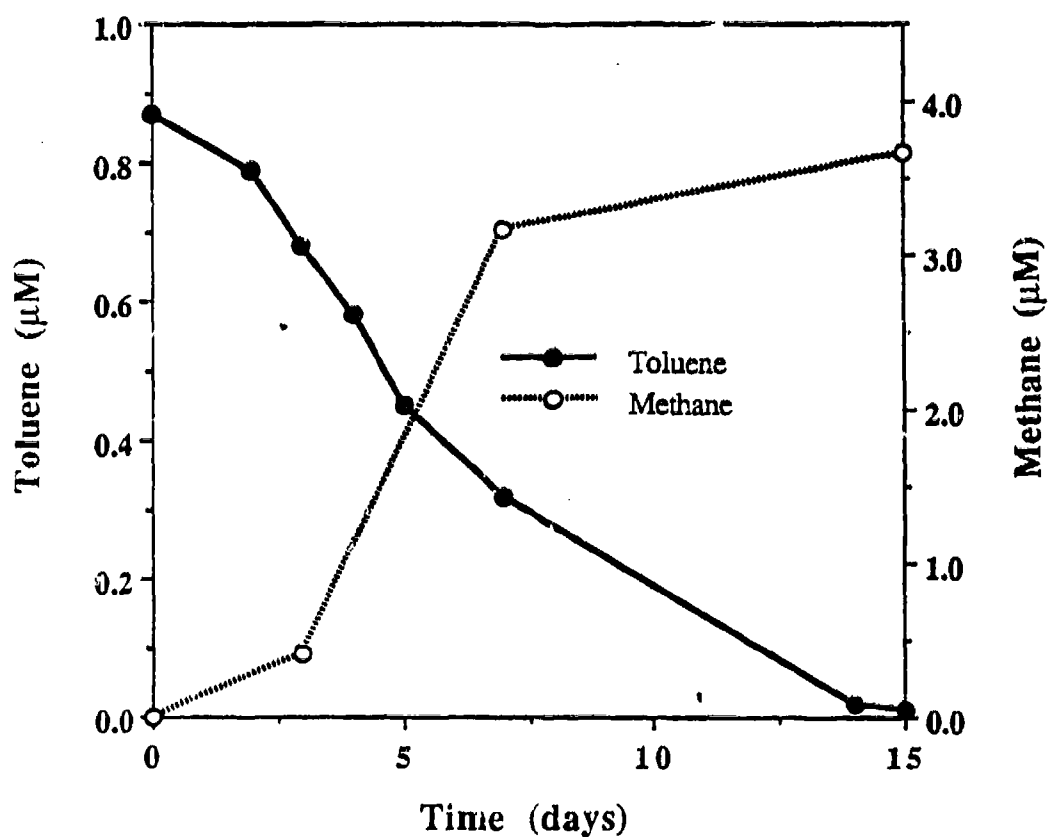


Figure 3. Stoichiometry of methane evolution and toluene degradation in a mixed methanogenic culture

## C. Stable Consortia

### 1. Characteristics

The consortia degrading toluene and *o*-xylene have been maintained on these compounds as sole carbon and energy sources for more than two years now. The consortia that are devoid of solids went through a transient period of slowing down of the degradation activity, whereas the solids-containing consortia continued degrading the hydrocarbons at a rapid rate (less than two weeks for a mixture of 5 mg L<sup>-1</sup> of each toluene and *o*-xylene). The slowdown of the activity in the absence of solids was perhaps due to a lack of support for microorganisms, or to shortage of some trace nutrient(s) or growth factor(s) available on aquifer solids. It had been shown before, by studying subsurface samples from the Pensacola aquifer, that most of the active microorganisms were attached to aquifer solids (Godsy et al., 1989 a). However, in these experiments, the solids-devoid enrichments took off after several months and gradually reached the same transformation rates as the solids-containing consortia. This interesting change may have resulted from the establishment of "mutual support" by microorganisms in the consortia (the bottles were not agitated, which allowed the microorganisms to stay in proximity of one another), or by multiplication of those members of the consortium that excreted growth factors stimulating other bacteria in the community.

The consortia were stained with acridine orange and observed with epifluorescence microscope. At least four different rod-shaped morphologies were visible in close association with each other. The cells were usually found clumped together or clumped onto a solid particle. The consortia were able to survive extended periods of time (at least one year) without any added carbon source. Upon refeeding, the cultures were slow to degrade toluene initially, but the activity was eventually completely restored. A culture that had been starved for one month and another culture that was actively degrading toluene were pasteurized at 80°C for 15 minutes. The cultures were cooled and re-fed toluene. Replicate pasteurized cultures also received an inoculum of methanogens previously enriched from the cultures (these methanogens could not degrade toluene on their own). Only the starved cultures remained active after pasteurization, with or without the addition of methanogens (Figure 4). This indicates that all the members of the community degrading toluene (including the methanogens) were able to withstand the heat shock of pasteurization, provided that the culture had been stressed beforehand to induce some form of

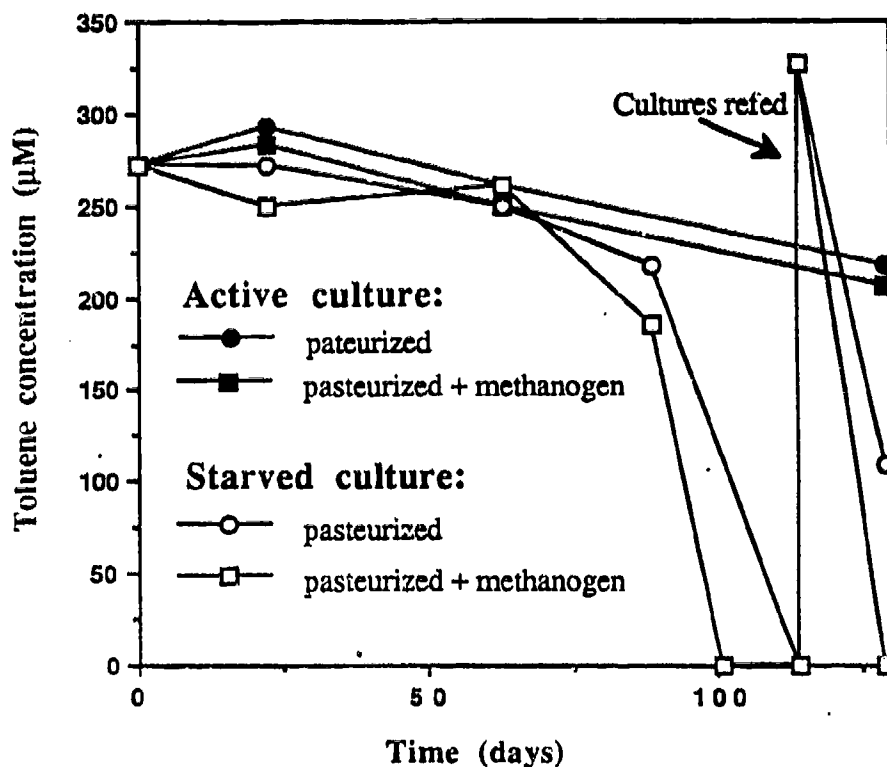


Figure 4. Effect of pasteurizing active and starved toluene-degrading methanogenic cultures. Cultures actively degrading toluene and cultures that had been starved for one month were heated to 80°C for 15 minutes, cooled, fed toluene, and in some cases spiked with methanogens previously enriched from the cultures. Only the starved cultures were able to withstand pasteurization with or without the addition of methanogens. The data are the mean of two replicates for each condition

sporulation. . The presence of spores was further confirmed by microcopic observation of the culture stained with malachite green while steaming (to stain endospores green), followed by staining with safranin to color the surrounding sporangium red. Spore formation certainly represents an advantage in subsurface microorganisms which are frequently exposed to starvation.

The optimum pH for the culture performance was pH 6; pH 7 seemed to be less favorable, and pH 8, the least (Figure 5). This is in accordance with the fact that pH of water in the Pensacola aquifer is 6 or below (E.M. Godsy, personal communication). The effect of temperature is shown in Figure 6. Somewhat surprisingly, the addition of exogenous electron acceptors, such as nitrate or sulfate, slowed down or completely inhibited the degradation of toluene (Figures 7 and 8). Oxygen was extremely toxic to these consortia (Figure 9). It can be concluded that the microbial communities in some zones of the Pensacola aquifer are best acclimated to the conditions of methanogenic fermentation. Sulfide also slowed down the transformation, which was to be expected since sulfide inhibits methanogenesis (Oremland, 1988). The methanogenic inhibitor BESA (2-bromoethanesulfonic acid) and the sulfate analog molybdate also inhibited toluene and *o*-xylene degradation (Figures 10 and 11). It was not surprising to find that BESA inhibited degradation since the consortium was methanogenic; however, molybdate is supposed to be a specific inhibitor of sulfate-reducers, yet no sulfate was added to the medium. The inhibition of toluene and *o*-xylene degradation by molybdate may indicate that sulfate-reducers were present as members of the consortium but that they were not reducing sulfate, or that molybdate was inhibiting some non-sulfate reducers in the cultures.

It was attempted to isolate pure cultures of MAH transformers from the consortia, using the anaerobic roll-tube method. Colonies were obtained after 3-4 weeks of incubation. Some of these colonies were transferred to liquid medium with toluene and *o*-xylene. Slow degradation of toluene was observed, but it was accompanied by methane production, indicating that the colonies were syntrophic associations of fermentative and/or acetogenic, and methanogenic bacteria. We did not succeed in separating the members of these syntrophic cultures, the likes of which are known to be based on very tight relationships (Mountfort and Bryant, 1982).

HPLC and GC/MS were used to try to detect initial transformation intermediates of toluene and *o*-xylene; however, probably due to low concentrations of the parent

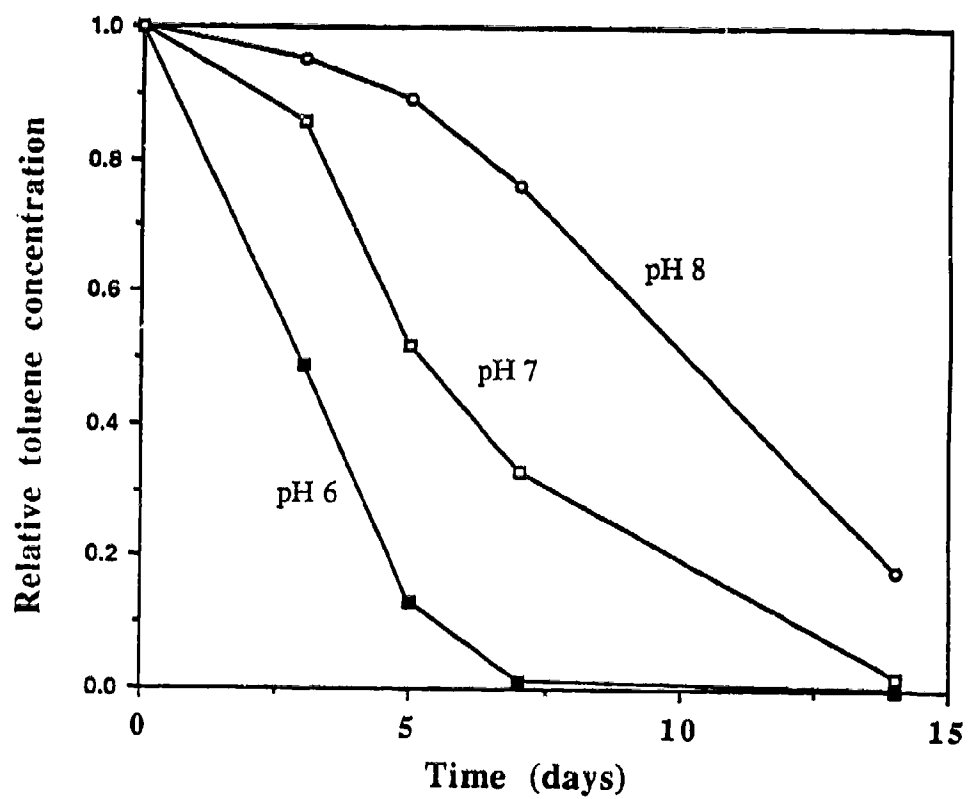


Figure 5. Effect of pH on toluene degradation in stable mixed methanogenic culture

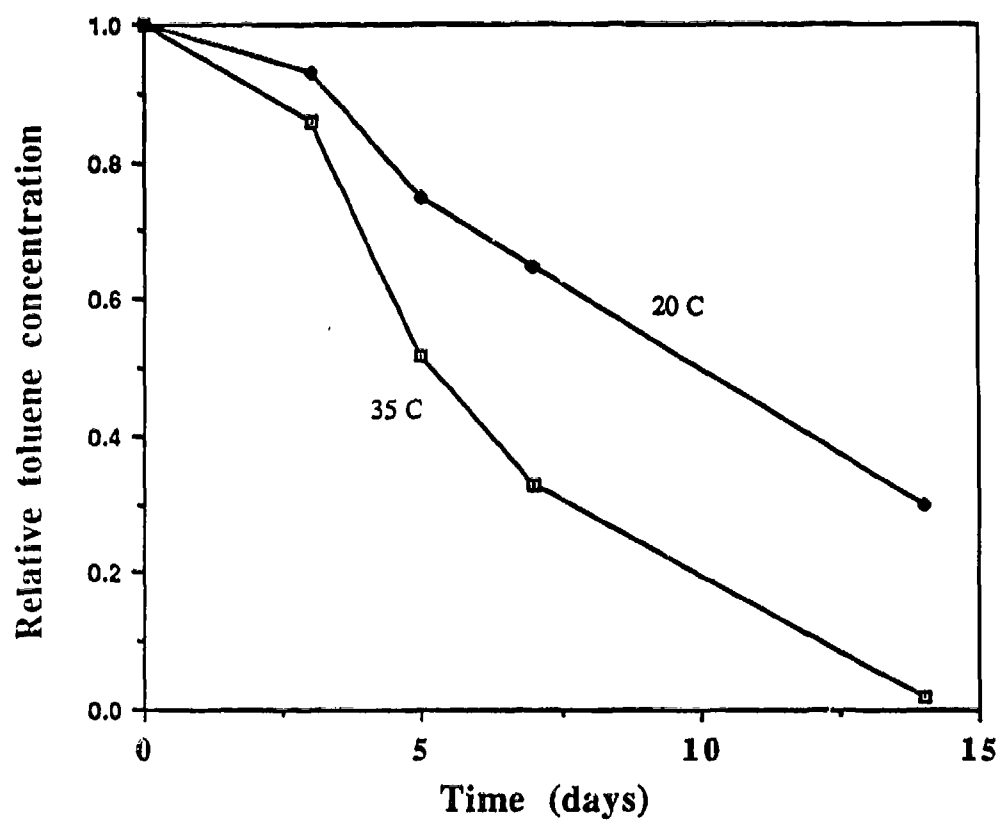


Figure 6. Effect of temperature on toluene degradation in stable mixed methanogenic culture

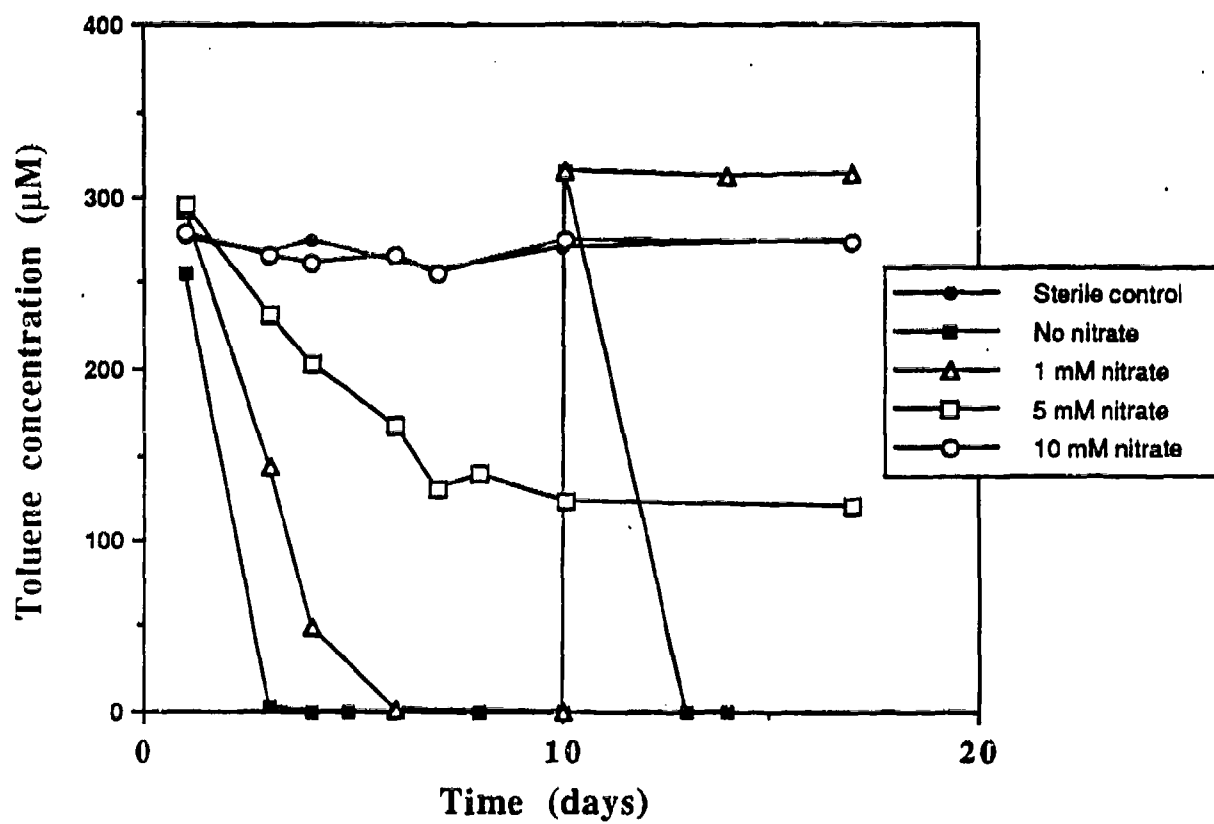


Figure 7. Effect of nitrate ( $\text{NaNO}_3$ ) on toluene degradation in stable mixed methanogenic culture



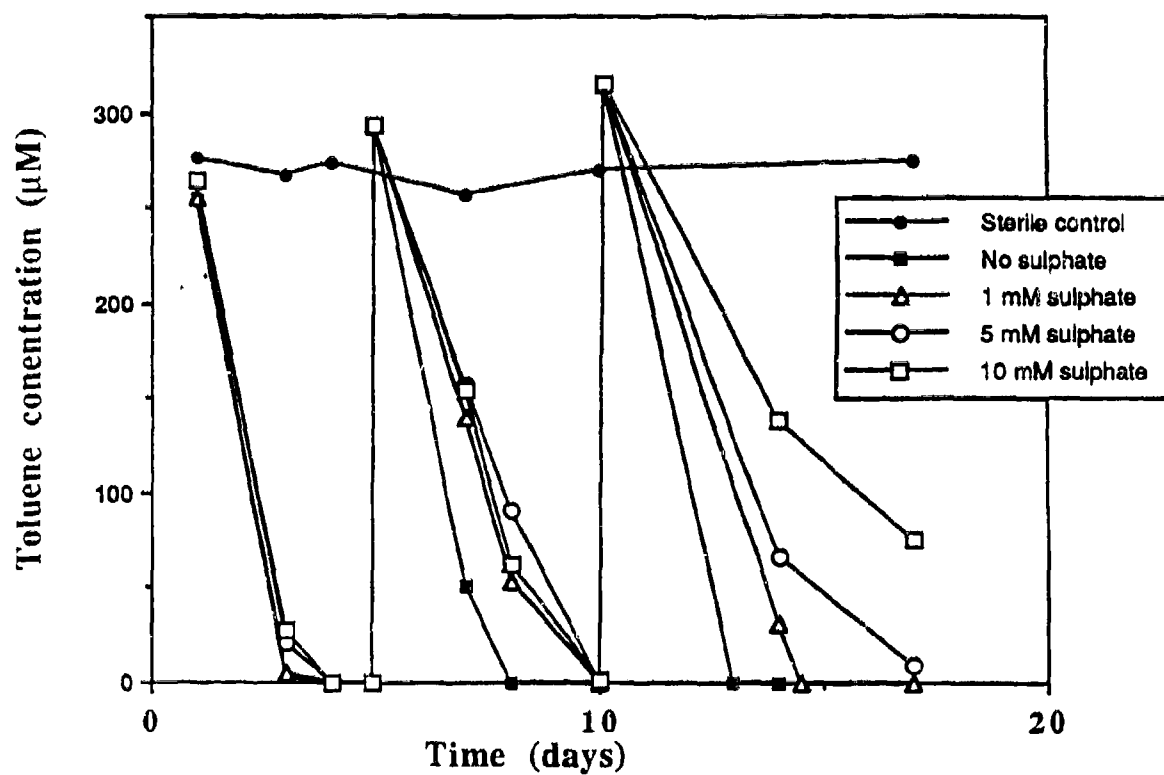


Figure 8. Effect of sulfate ( $\text{NaSO}_4$ ) on toluene degradation in stable mixed methanogenic culture

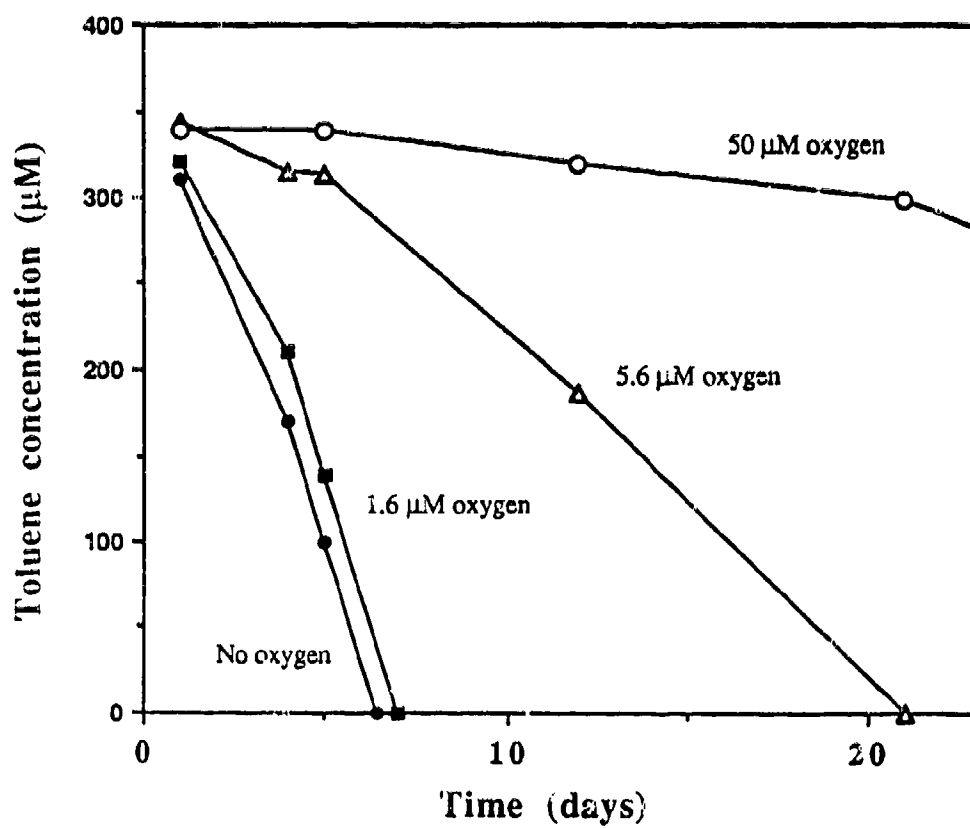


Figure 9. Effect of Oxygen ( $\text{O}_2$ ) on toluene degradation in stable mixed methanogenic culture

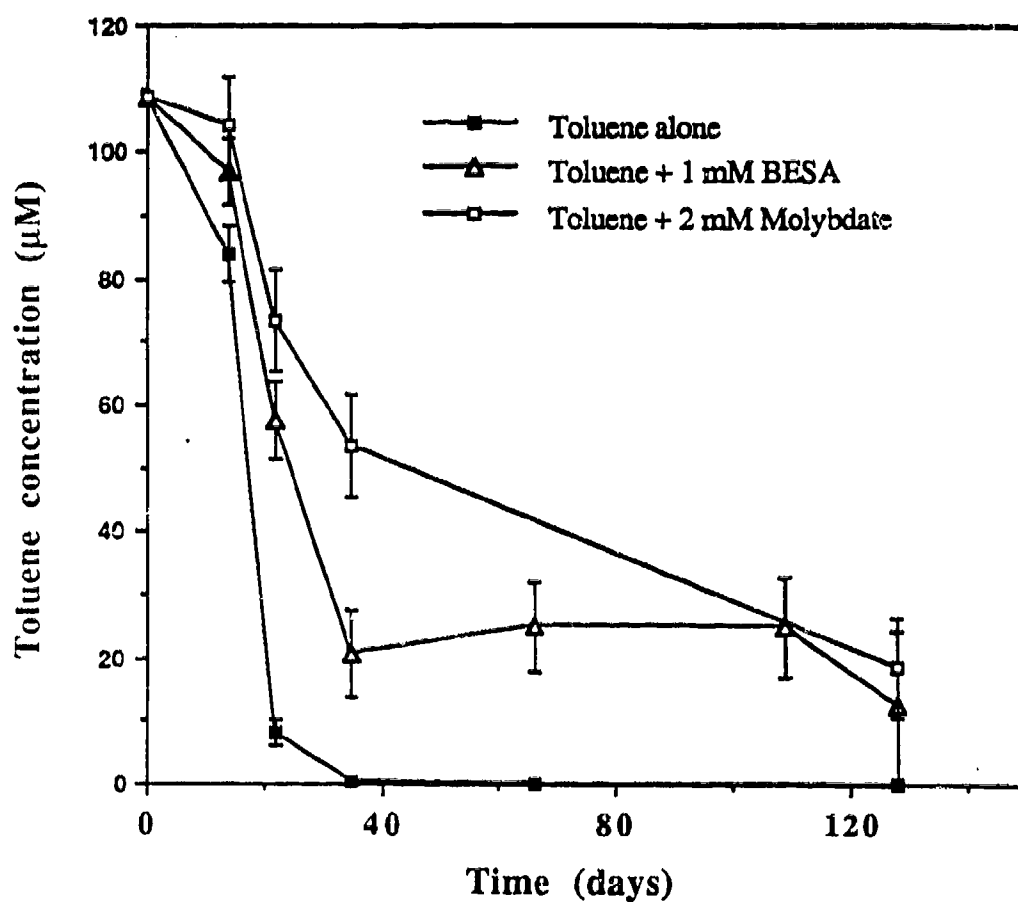


Figure 10. Effects of 2-bromoethanesulfonic acid (BESA) and molybdate ( $\text{MoO}_4$ ) on toluene degradation in stable mixed methanogenic culture

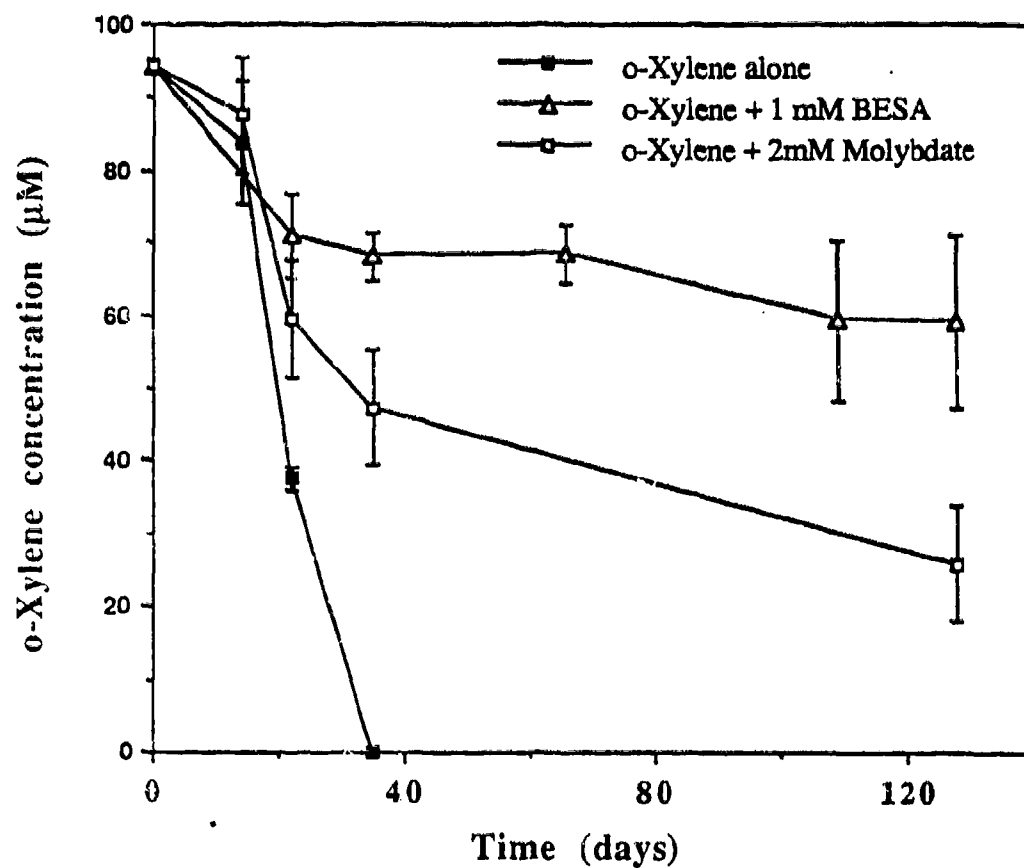


Figure 11. Effects of 2-bromoethanesulfonic acid (BESA) and molybdate ( $\text{MoO}_4$ ) on *o*-xylene degradation in stable mixed methanogenic culture

compounds used, these attempts were unsuccessful. Acetate was detected as a transient intermediate (maximum concentration 0.07 mM) and would build up in BESA-inhibited cultures up to 0.3-0.4 mM. The simultaneous adaptation method (Stanier, 1947) was utilized in an attempt to elucidate the initial pathway of toluene oxidation. Benzyl alcohol, benzaldehyde, and benzoate (possible intermediates if methyl hydroxylation were the first step), and the cresol isomers (possible intermediates if ring hydroxylation occurred) were tested as substrates. All were degraded without a lag except *o*-cresol (slight lag) and *m*-cresol (not degraded). These results gave little insight into the actual pathway (i.e., ring- versus methyl-hydroxylation). It is very possible that in a mixed culture such as this, both pathways are operative at variable levels of importance. Table 4 lists some compounds that were or were not degraded by the toluene-degrading methanogenic culture.

The enzymes involved in the degradation of toluene and *o*-xylene appear to be substrate-specific. The cultures readily degraded toluene and *o*-xylene, but not *m*- or *p*-xylene (Figure 12). Enrichments fed both toluene and *o*-xylene degraded both compounds simultaneously with no evidence of competition. In addition, enrichments fed only toluene for over two years could no longer degrade *o*-xylene, and vice-versa, suggesting that the enzymes responsible for the initial attack on the substrates toluene and *o*-xylene are not the same (Figure 13).

## 2. Kinetics

The doubling time for the stable mixed cultures utilizing toluene as sole source of carbon and energy was 6-10 days. Because the growth was so slow, the rate of toluene degradation depended strongly on the initial biomass concentration (Figure 14). This point is also demonstrated by the observation that over a period of two years, the rate of degradation in the same enrichment culture increased ten-fold predominantly as a result of increased biomass concentration (Figure 15). The data from substrate depletion curves for toluene and *o*-xylene were fit to the Monod kinetic model by non-linear regression (assuming no cell decay). The results are shown in Figures 16 and 17. The yield ( $Y$ ) and initial biomass concentration ( $X_0$ ) were estimated from protein measurements assuming 50% of a cell's dry weight was protein. The half saturation constant ( $K_s$ ) and the maximum specific growth rate ( $\mu_{max}$ ) were estimated by non-linear regression of the data to the Monod model. At initial substrate concentrations higher than those shown in Figures 16 and 17, the data no longer fit

Table 4. Substrate screen

DEGRADED to CH <sub>4</sub> and CO <sub>2</sub>	NOT DEGRADED
-----	-----
toluene	benzene
<i>o</i> -xylene	<i>m</i> -, <i>p</i> -xylene
<i>p</i> -, <i>o</i> -cresol	ethylbenzene
benzylalcohol	naphthalene
benzaldehyde	cyclohexane
benzoate	methylcyclohexane
fatty acids (C3-C7)	phenol
casamino acids	<i>m</i> -cresol
yeast extract	
glucose	
methanol	
acetone	
acetate	
formate	
hydrogen	

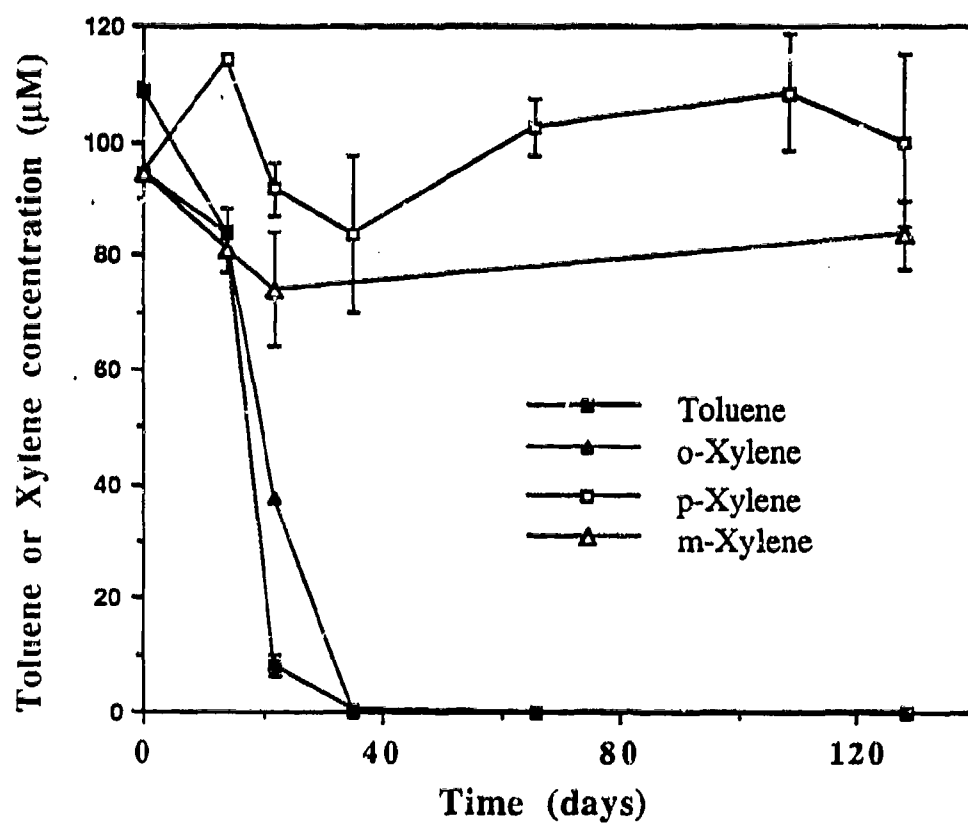


Figure 12. Substrate specificity 1. Only toluene and *o*-xylene were degraded under the experimental conditions used. *m*-Xylene and *p*-xylene were not degraded

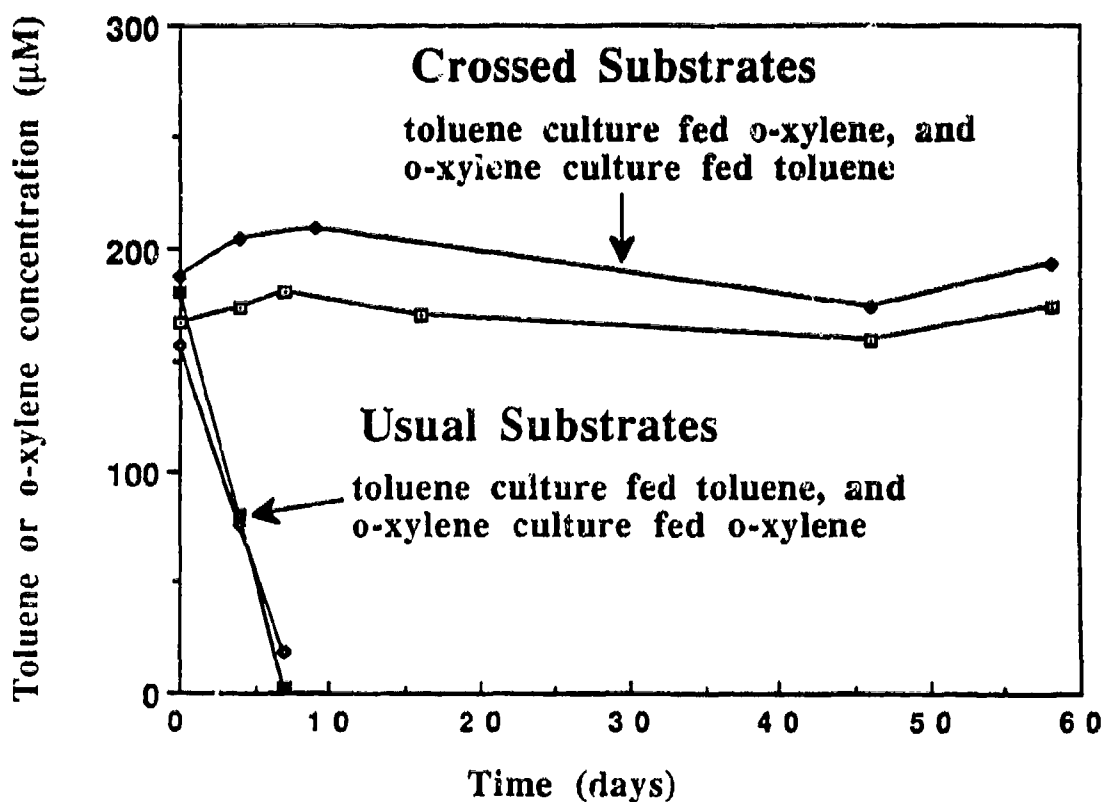


Figure 13. Substrate specificity 2. An enrichment culture fed only toluene for over two years no longer degraded o-xylene; similarly an enrichment culture fed o-xylene for over two years no longer degraded toluene



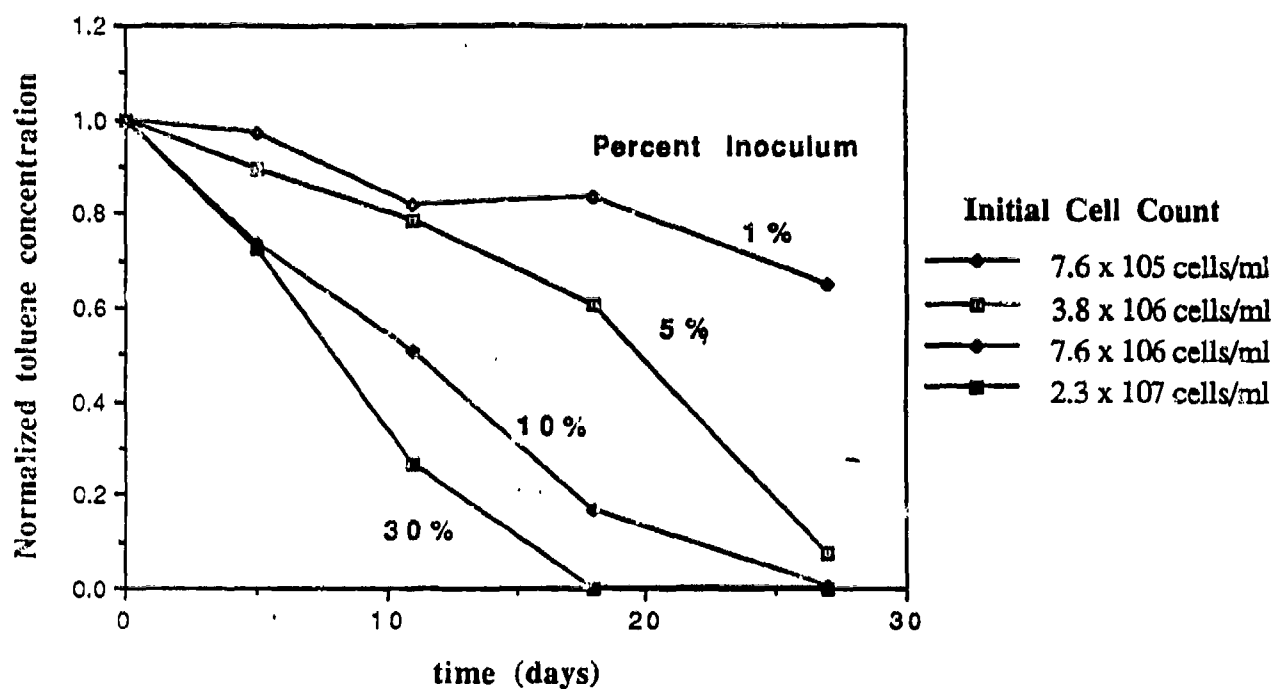


Figure 14. Effect of initial biomass concentration on the rate of toluene degradation in stable mixed methanogenic culture

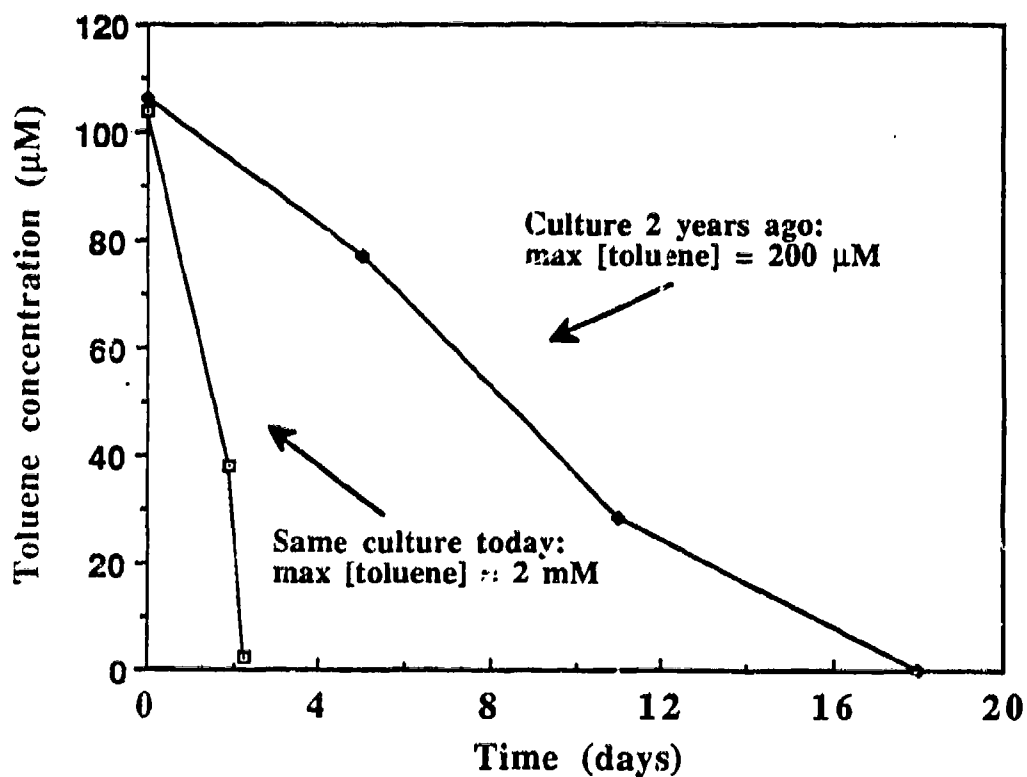


Figure 15. Enrichment of toluene- and *o*-xylene-degrading activity.

Over the past two years, the apparent rates of toluene and *o*-xylene degradation increased ten-fold. The cultures also became tolerant of much higher substrate concentrations

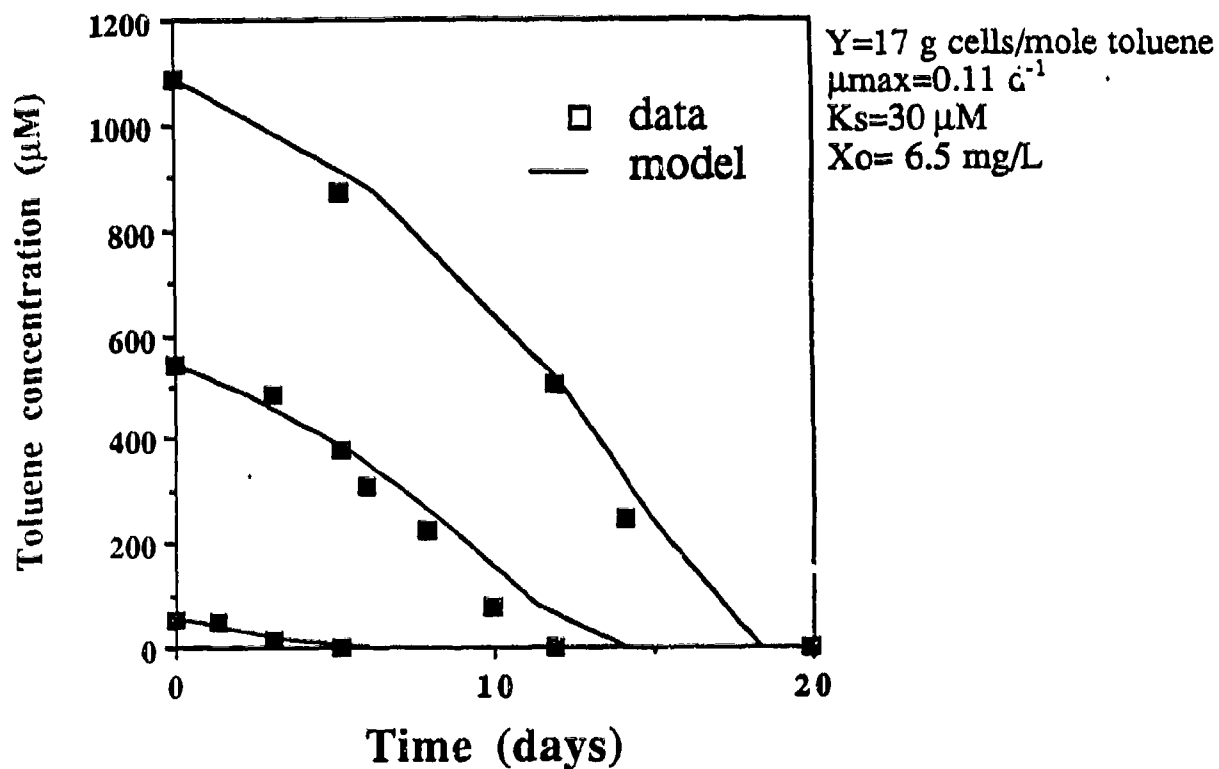


Figure 16. Kinetics of toluene degradation. Experimental data (symbols) are plotted along with Monod kinetic model predictions (solid lines). The biomass yield ( $Y$ ) and the initial biomass concentration were measured experimentally. The Monod parameters  $K_s$  (half-saturation constant) and  $\mu_{\max}$  (maximum specific growth rate) were approximated by non-linear regression of the data from substrate depletion curves to the Monod model

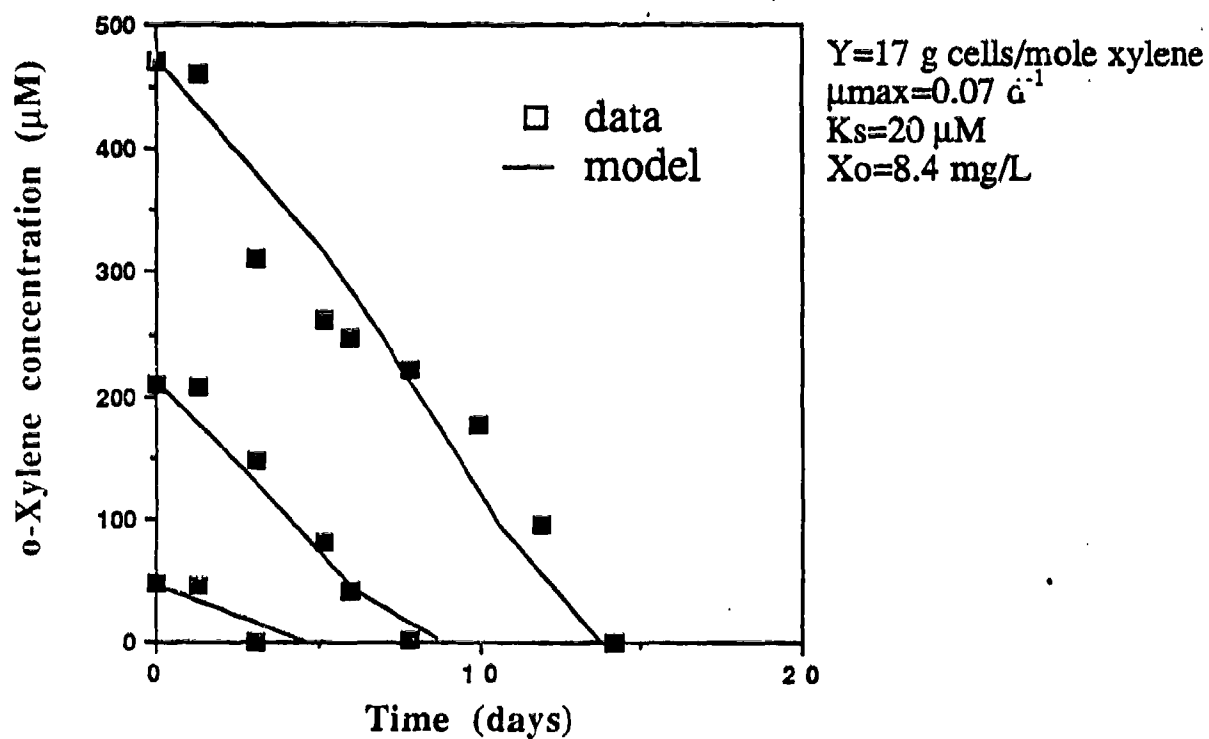


Figure 17. Kinetics of *o*-xylene degradation. Experimental data (symbols) are plotted along with Monod kinetic model predictions (solid lines). (See Figure 16 for details)

the model at all. The rates of degradation slow down considerably because the substrates toluene and *o*-xylene are toxic at high concentrations (see Figure 18).

### **3. Effects of various substances on toluene degradation**

To determine if the presence of carbon sources other than toluene would stimulate or inhibit toluene degradation, a variety of test compounds were fed to the consortia in addition to toluene. Both the test compound and toluene were fed simultaneously. In each case, the initial toluene concentration remained constant while the initial concentration of the test compound varied over two orders of magnitude. The compounds tested and a brief summary of the effects are listed in Table 5. The results are also depicted for selected compounds in Figures 19 to 25. Overall, none of the compounds tested stimulated degradation of toluene. Most of the compounds tested were utilized by the consortia as growth substrates preferentially over toluene. Only when the test compound was nearly completely degraded did the degradation of toluene really begin. Acetate and  $H_2$  inhibited toluene degradation most likely because these compounds are intermediates in the degradation of toluene, and if their concentrations are elevated, the reactions leading to the formation of acetate or hydrogen from toluene are no longer energetically favorable and the degradation of toluene stops. In the case of more complex substrates such as glucose, fatty acids, and casamino acids, acetate was observed to build up during the degradation of these compounds, which in turn would block toluene metabolism. Methanol was immediately utilized by methanogens, and while methanol was being utilized, toluene degradation did not proceed, probably because the methanogens were selectively utilizing methanol over any acetate produced from toluene. Acetone had initially no effect on toluene degradation because acetone was being utilized only very slowly by the cultures. However, after all the toluene and acetone from the first feeding had been consumed, the cultures were refed, and this time, acetone degradation began immediately (presumably because the cultures had adapted to utilize this substrate) and blocked toluene degradation much like in the case of methanol. These findings have a great significance for understanding and predictions of degradation processes in the field: when mixtures of contaminants (or contaminants and natural substrates) are present, which is most frequently the case, the anaerobic degradation of aromatic hydrocarbons may be delayed or completely precluded.

Some of the compounds tested were toxic and were not metabolized. This was the case with alkanes, cyclohexane, and carbon tetrachloride. The aromatic

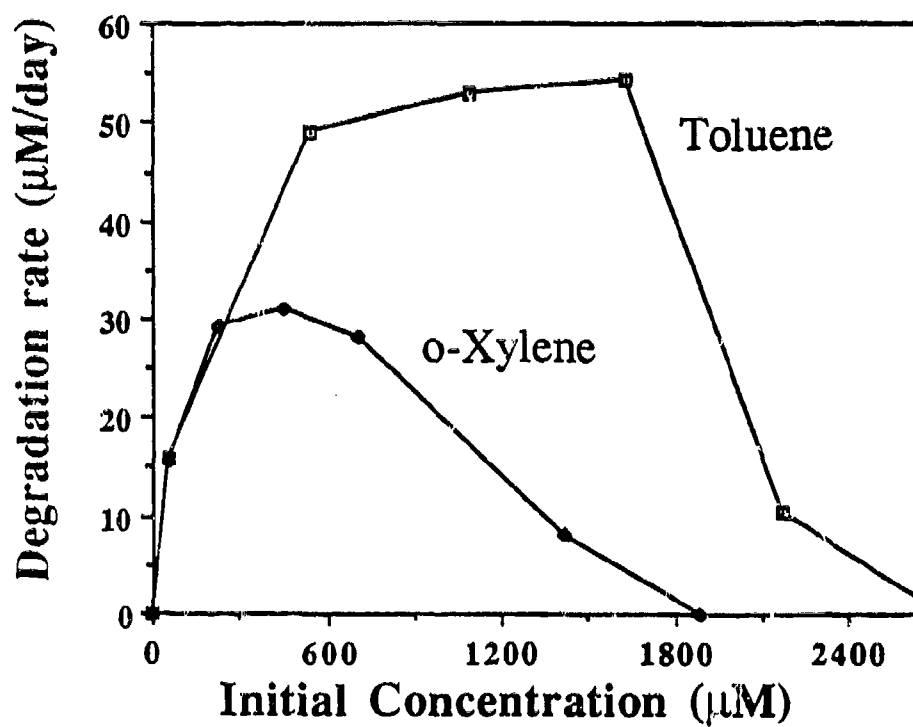


Figure 18. Rate of degradation versus initial substrate concentration in mixed methanogenic cultures enriched on either toluene or o-xylene

Table 5. Effects of various organic compounds on toluene degradation

SUBSTANCE	EFFECT OF SUBSTANCE ON TOLUENE DEGRADATION
Acetate	<p>Toluene degradation was inhibited at acetate concentrations from 0.5 -2.0 mM (see Figure 19).</p> <p>Toluene degradations begins once acetate is consumed. Since acetate is an intermediate in toluene degradation, the inhibition can be explained from a thermodynamic point of view: at high acetate concentrations, the conversion of toluene to acetate is no longer energetically favorable.</p>
Hydrogen	<p>70 % H<sub>2</sub> in the headspace weakly inhibits toluene degradation. As in the case of acetate, H<sub>2</sub> is a likely intermediate of toluene degradation, and therefore the inhibition is probably a result of unfavorable energetic situation.</p>
Methanol	<p>Inhibits toluene degradation at methanol concentrations from 0.2 - 2.0 mM.</p> <p>Methanol was rapidly consumed by methanogens. In this case, methanol was not a likely intermediate in toluene degradation. However, it was a very good substrate for the methanogens and was utilized preferentially over acetate and H<sub>2</sub> coming for toluene breakdown.</p>
Glucose	<p>Inhibits toluene degradation at glucose concentrations starting at 170 <math>\mu</math>M (see Figure 20).</p> <p>Glucose was rapidly metabolized by the mixed culture producing large amounts of methane and containing high numbers of large bacilli. In this case, acetate built up during the degradation of glucose and it is likely that in turn, the acetate produced inhibited toluene degradation as explained in the first paragraph.</p>
Propionate	<p>Inhibits toluene degradation at propionate concentrations starting at 290 <math>\mu</math>M (see Figure 21).</p> <p>Propionate was converted to methane more slowly than was acetate. As for glucose, the inhibition was most likely a result of transient acetate buildup.</p>
Fatty acids (C4 to C6, n- and iso-)	<p>Inhibit toluene degradation at total fatty acids concentrations starting at 150 <math>\mu</math>M (see Figure 22). Again, the inhibition was most likely a result of transient acetate buildup</p>
Casamino acids	<p>Inhibit toluene degradation at casamino acid concentrations starting at 150 <math>\mu</math>M (see Figure 23). Again, the inhibition was most likely a result of transient acetate buildup</p>
Yeast Extract	<p>Inhibits toluene degradation probably for the same reasons as above</p>
Cysteine-HCl	<p>Inhibits toluene degradation probably for the same reasons as above</p>

- Acetone Initially, acetone had no effect on toluene degradation (see Figure 24). During this period, acetone was not degraded. However, after a short adaptation period, the culture metabolized acetone to  $\text{CH}_4$  and  $\text{CO}_2$ . Subsequently, acetone and toluene were refed to the culture, and this time acetone strongly inhibited toluene degradation because it was immediately consumed.
- Cyclohexane At concentrations below  $140\ \mu\text{M}$ , no inhibition of toluene degradation occurred. At concentrations above  $700\ \mu\text{M}$ , complete inhibition occurred due to toxicity (see Figure 25). Cyclohexane (and other cycloalkanes and alkanes) were not degraded by the culture, but at higher concentrations these types of compounds are known to dissolve into and destroy cell membranes.
- Carbon tetrachloride Very toxic. Toluene degradation completely inhibited and methanogenesis from acetate completely inhibited at  $40\ \mu\text{M}$  carbon tetrachloride.



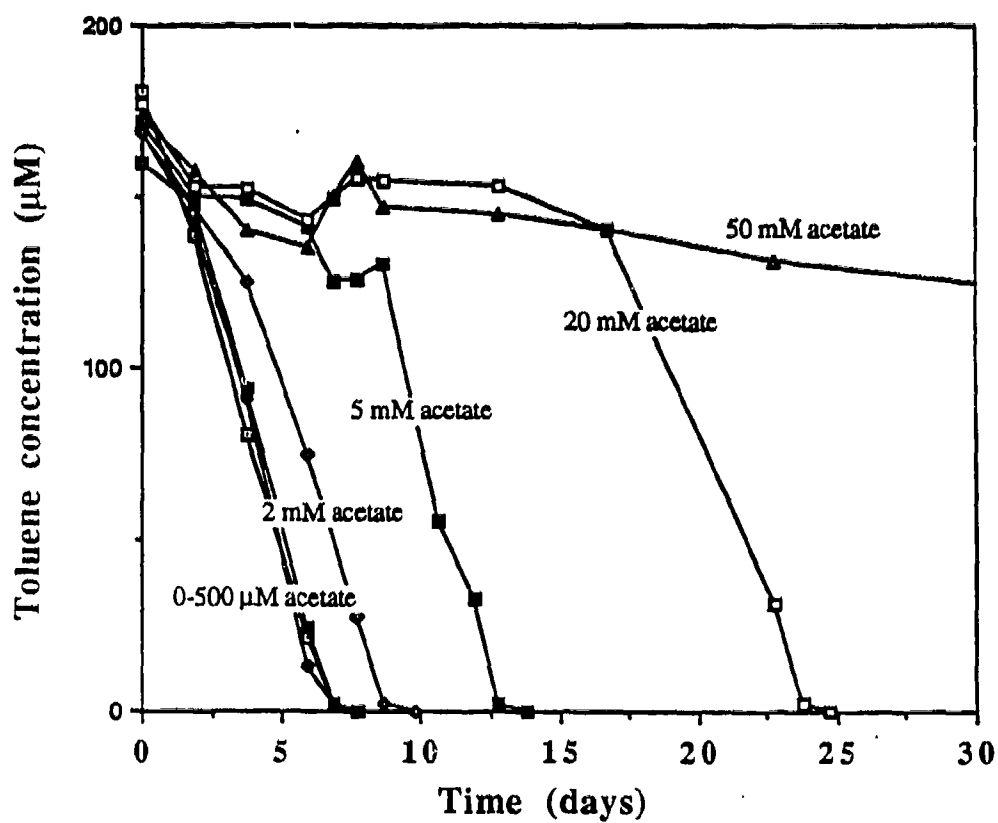


Figure 19. Inhibition of toluene degradation by acetate

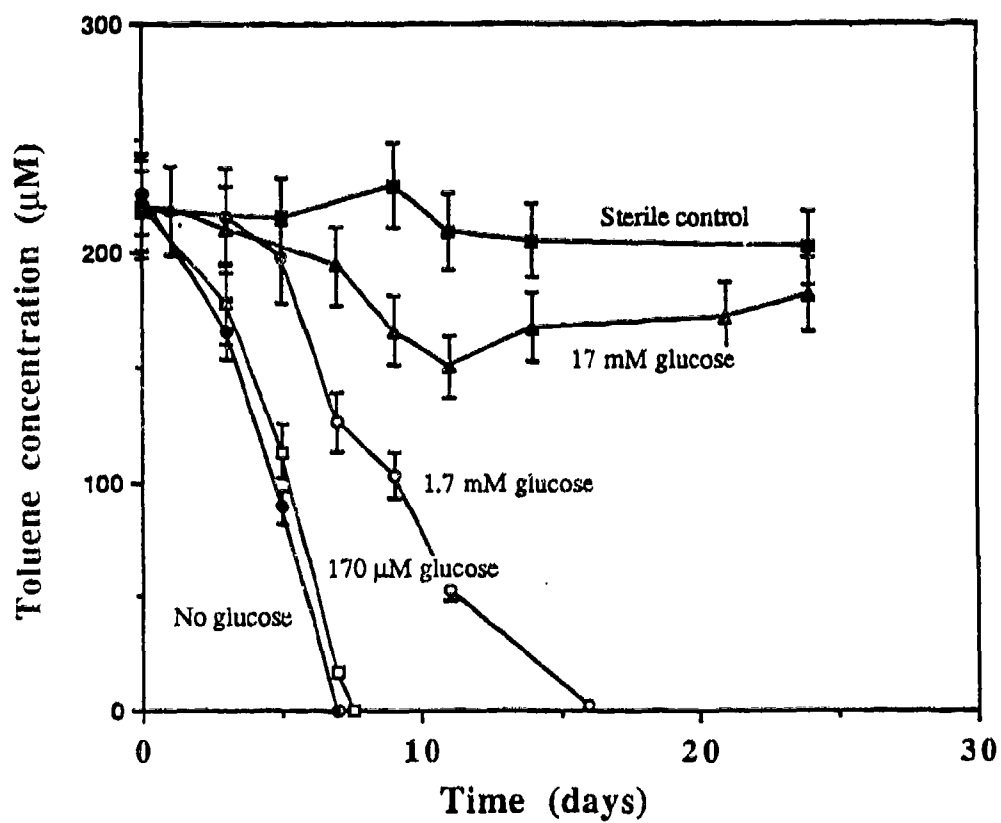


Figure 20. Inhibition of toluene degradation by glucose

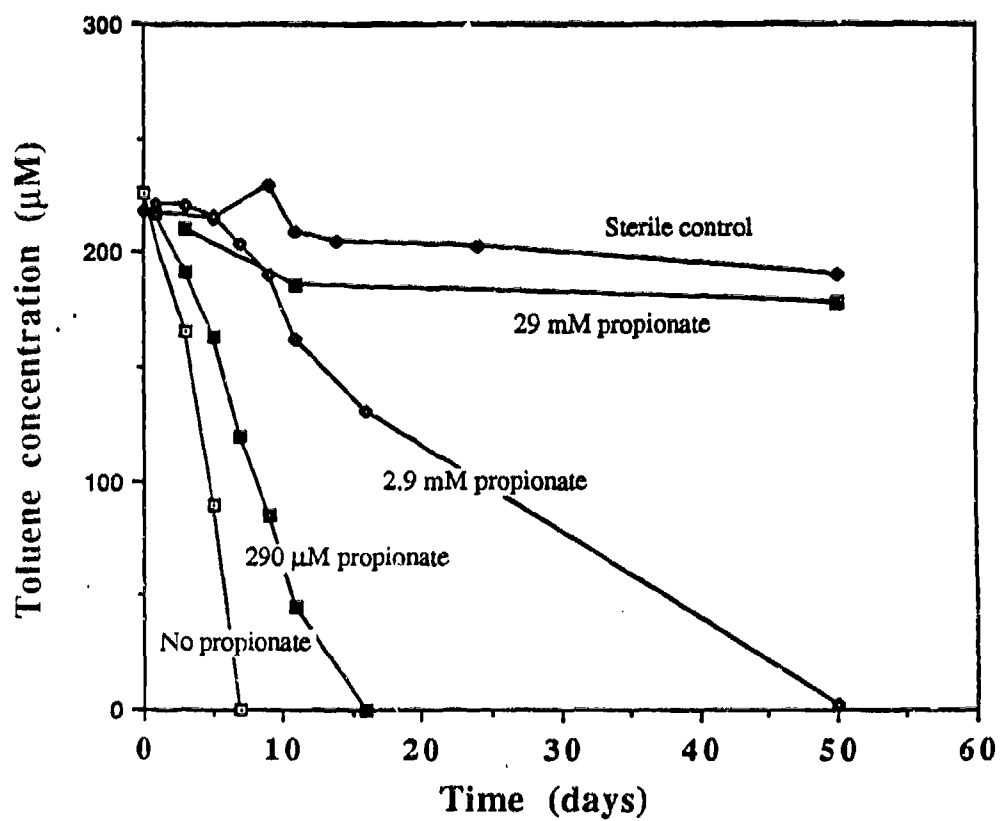


Figure 21. Inhibition of toluene degradation by propionate

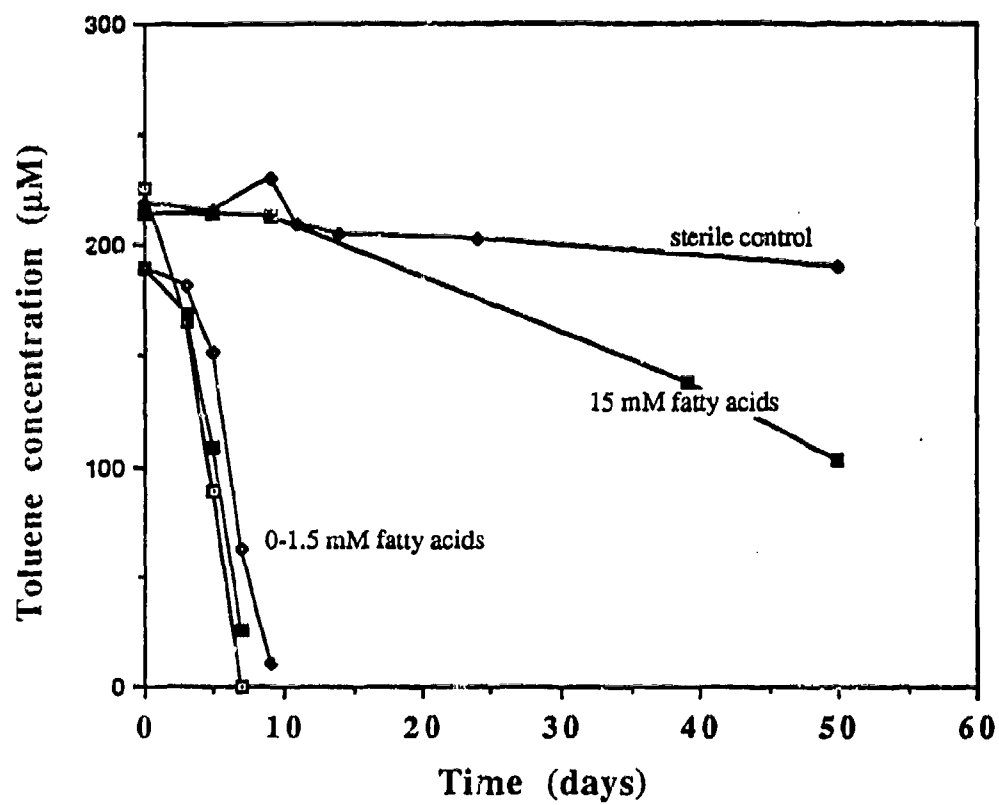


Figure 22. Inhibition of toluene degradation by fatty acids

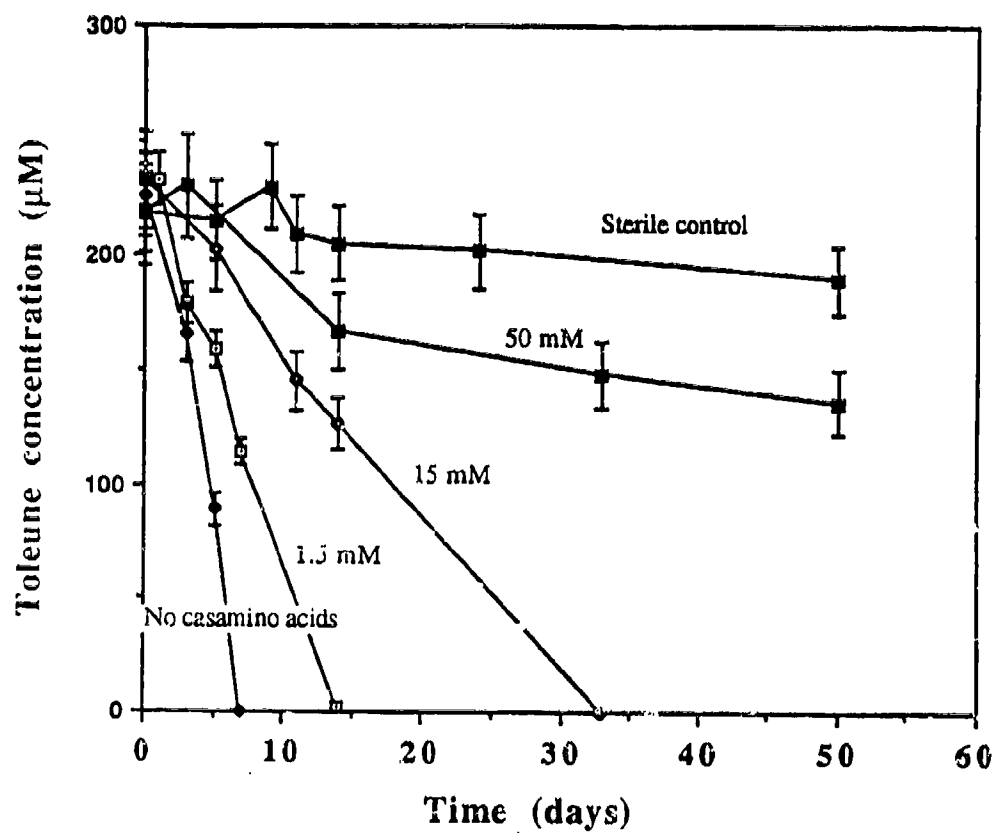


Figure 23. Inhibition of toluene degradation by casamino acids

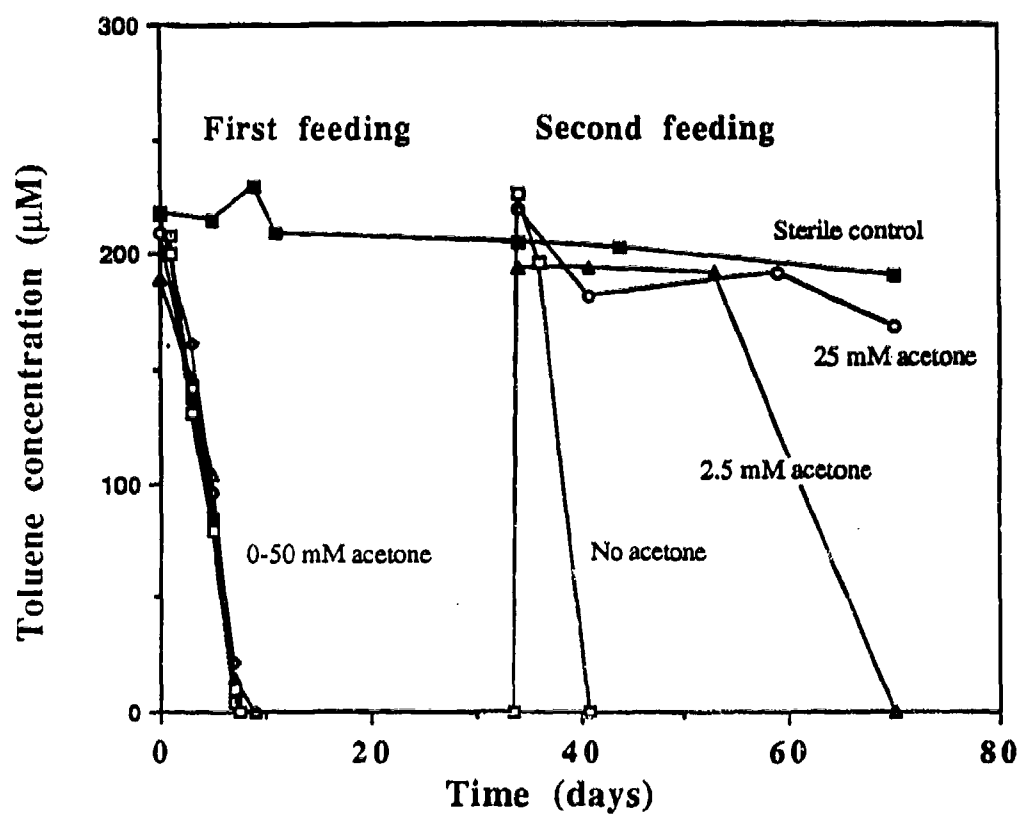


Figure 24. Effect of acetone on toluene degradation

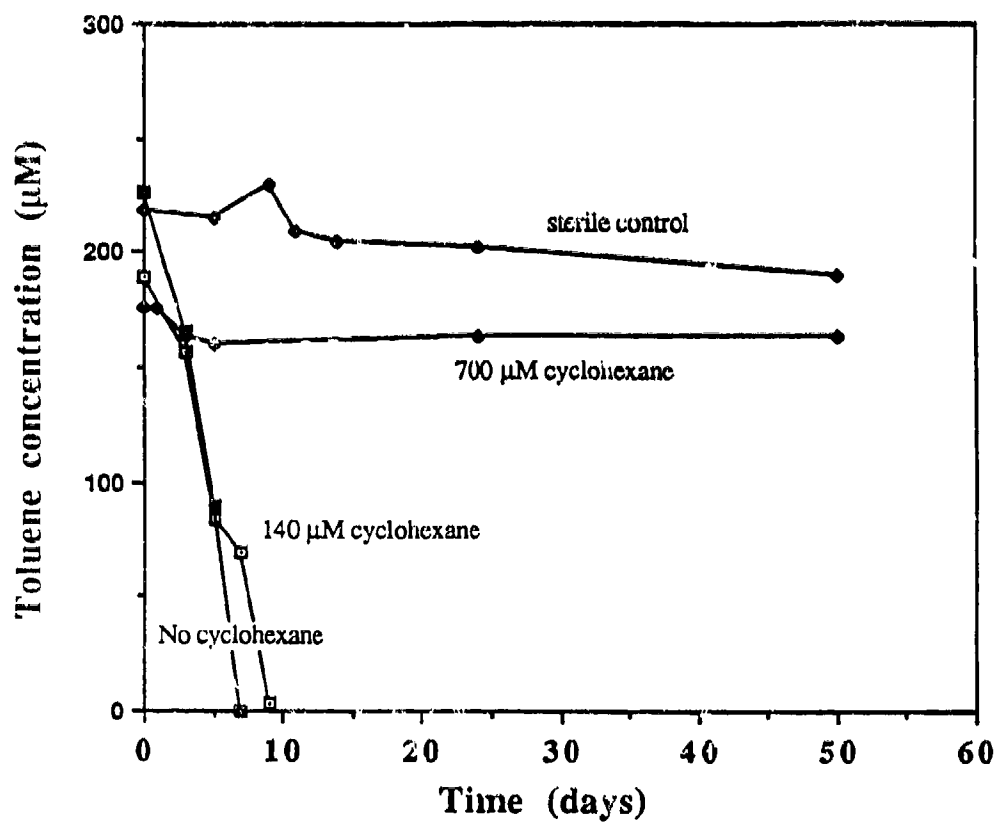


Figure 25. Effect of cyclohexane on toluene degradation

hydrocarbons themselves, including toluene and *o*-xylene, were also toxic at certain concentrations. The toxicity of toluene and *o*-xylene is demonstrated in Figure 18. In addition, the toxic effect of toluene and *o*-xylene was dependent on the length of time of exposure to these compounds. A similar effect was also demonstrated for another aromatic hydrocarbon, benzene. The rate of toluene or *o*-xylene degradation as a function of time of exposure to benzene was observed in cultures fed benzene in addition to their normal substrates, toluene or *o*-xylene (Figure 26). In the cultures that were not amended with benzene, the rate of toluene or *o*-xylene degradation increased with time, but in the cultures amended with benzene, the rate of toluene or *o*-xylene degradation decreased all the way to zero after 50-100 days of incubation in the presence of benzene.

The maximum concentration of toluene or *o*-xylene that could be degraded by the cultures depended on the initial cell density. At higher cell densities, higher initial toluene or *o*-xylene concentrations were tolerated because the rate of degradation was faster and hence the length of time of exposure to high concentrations of the substrate was shorter. The cultures would survive because the growth rate exceeded the decay caused by substrate toxicity. However, if a small inoculum was used together with high initial substrate concentration, the cultures would frequently die of toxic exposure before being able to degrade the substrate sufficiently to reduce its concentration to less toxic levels.



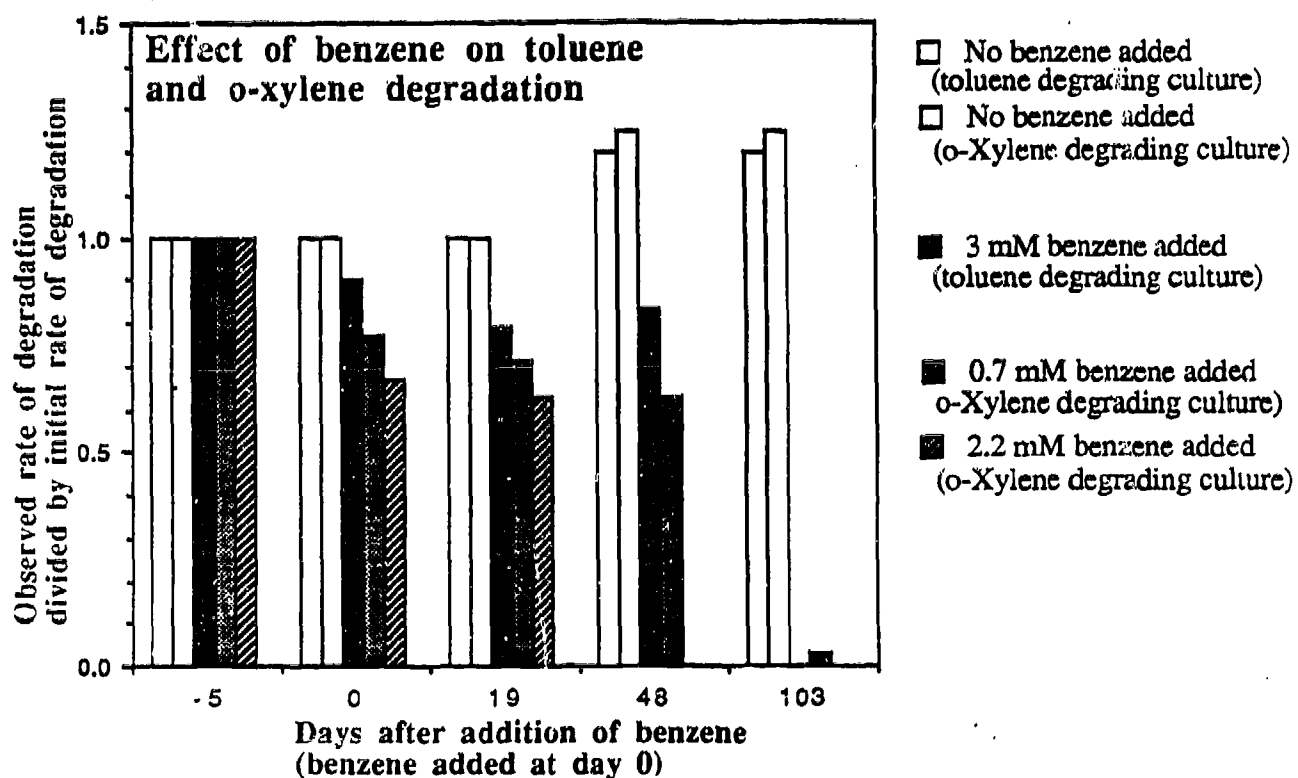


Figure 26. Effect of benzene on the rate of toluene and o-xylene degradation. Initially benzene has only a slight inhibitory effect. Longer exposures to benzene significantly decrease the rate of degradation

## SULFATE-REDUCING DEGRADATION OF MAH

Aquifer samples from Seal Beach (CA) were used to set up microcosms under sulfate-reducing conditions (25 mM sodium sulfate added). The gasoline-contaminated Seal Beach site naturally contains relatively high concentrations of sulfate because of its proximity to an intertidal marsh; therefore, sulfate-reducing degradation of hydrocarbons was expected to occur. The microcosms were amended with a mixture of benzene, toluene, ethylbenzene, *o*-xylene, and *p*-xylene, each in concentration of approximately 5 mg L<sup>-1</sup>. Toluene was more than 80% degraded by day 40; it was followed by *p*-xylene (by day 72), and then *o*-xylene (by day 104; Figure 27). The relatively short adaptation times observed may be due to the pre-exposure of the sediments to aromatic hydrocarbons. It is highly likely that these degradation processes also occur in the site naturally. Neither benzene nor ethylbenzene were degraded during 270 days of incubation. No transformation of any of the compounds occurred in autoclaved controls. After this initial adaptation period, toluene, *p*-xylene, and *o*-xylene were refed and degradation proceeded without a lag.

Sulfate concentration decreased and a black precipitate formed only in active microcosms (presumably as a result of hydrogen sulfide formation and FeS precipitation). Figure 28 shows the dependence of toluene degradation on the presence of sulfate in microcosms; the degradation of the hydrocarbon ceased when sulfate became depleted and resumed upon addition of sulfate. The same effects were observed for *p*- and *o*-xylene. Molybdate (2 mM), a known inhibitor of sulfate reduction, completely inhibited toluene degradation, whereas 1 mM 2-bromoethanesulfonic acid (BESA), an inhibitor of methanogenesis, did not (Figure 29).

Primary enrichments inoculated with both culture fluid and a small amount of solids from active microcosms into prereduced defined mineral medium retained the degradative activity towards toluene, *p*-, and *o*-xylene. These enrichments also degraded *m*-xylene without a lag. From these cultures, only the liquid portion was transferred as inoculum to prepare secondary enrichments; the activities were retained. The secondary enrichments (suspended cultures) without solids were used for mass balance experiments because the effects of sorption and unknown carbon sources and acceptors in the sediments were minimized. Table 6 shows the measured amounts of sulfate consumed and sulfide produced per mole of toluene or

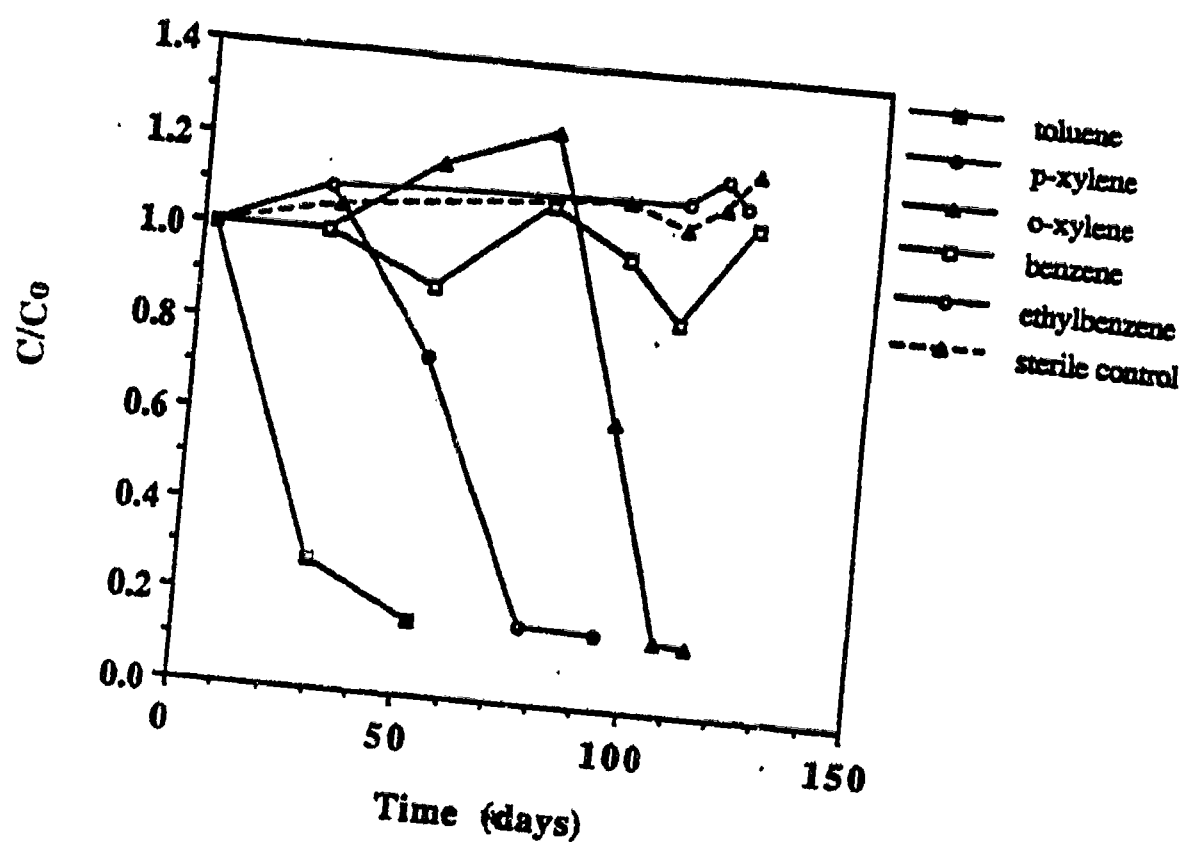


Figure 27. Degradation of toluene, *p*-xylene, and *o*-xylene in unacclimated microcosms. The vertical axis is concentration normalized by dividing the concentration at a given time by the initial concentration of that substrate. The results for active microcosms are the mean of six replicates. The results for autoclaved controls are the mean of two replicates with all five hydrocarbons. The concentrations of toluene, *p*-xylene, and *o*-xylene never decrease to zero because of desorption from the sediments (these sediments sorb MAH compounds strongly)

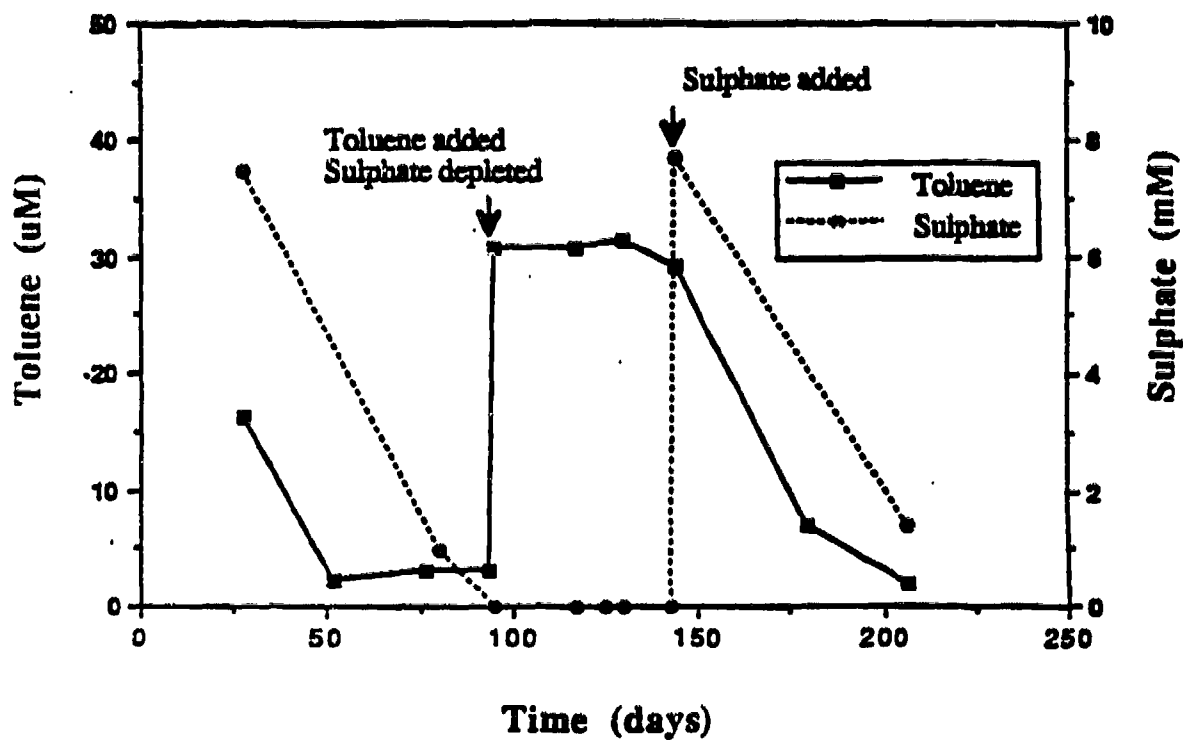


Figure 28. Effect of sulfate on toluene degradation in microcosms. Degradation proceeds as long as sulfate is present, ceases when sulfate becomes depleted, and resumes upon addition of sulfate. The data shown were taken from one microcosm only although the trends are representative of all six active microcosms

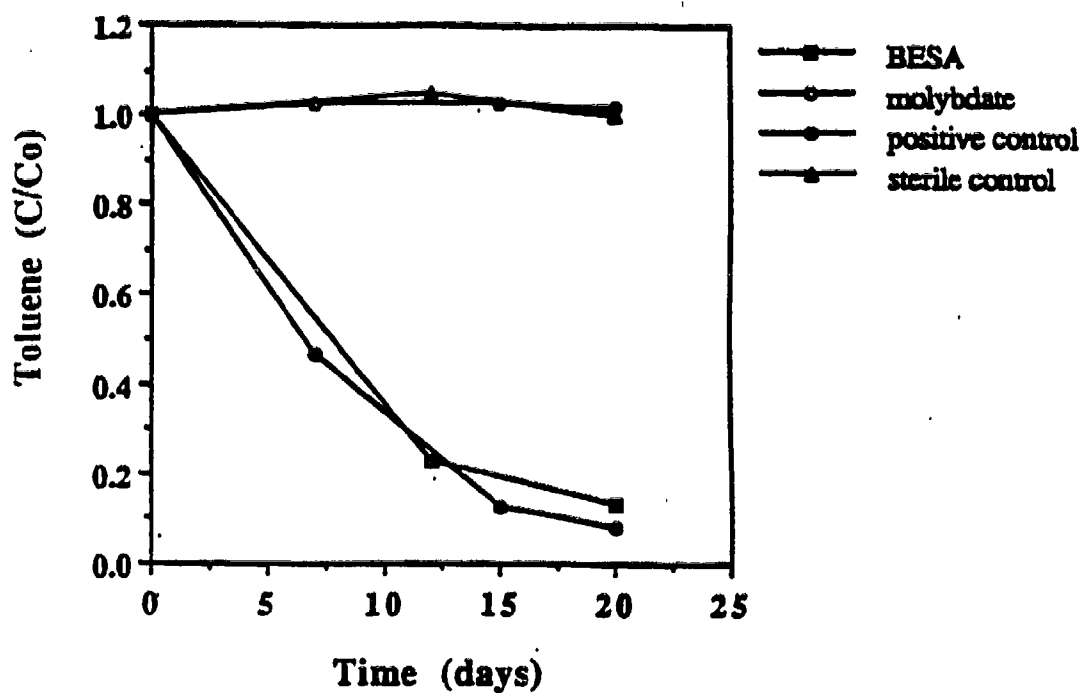


Figure 29. Effect of BESA (1 mM) and sodium molybdate (2 mM) on toluene degradation in enrichment cultures. Positive controls received neither BESA nor molybdate. The results are the mean of two replicates

Table 6. Measured and theoretical amounts of sulfate consumed and sulfide produced (in moles) per mole of toluene or *m*-xylene consumed<sup>a</sup>

Substrate	Sulfate Consumed <sup>b</sup>	Sulfide Produced <sup>b</sup>
<b>Toluene</b>		
(measured)	$4.5 \pm 0.3$	$1.6 \pm 0.7$
(theoretical)	4.14	4.14
<b><i>m</i>-Xylene</b>		
(measured)	$4.9 \pm 0.4$	$4.1 \pm 1.0$
(theoretical)	4.83	4.83

<sup>a</sup> The theoretical amounts have been calculated according to McCarty (1971), assuming an efficiency of energy transfer of 60%..

<sup>b</sup> Values are mean  $\pm$  standard deviation of 5 or 6 replicates.

*m*-xylene degraded in suspended cultures, in comparison with the theoretical stoichiometric ratios. It needs to be emphasized that the method used for sulfide measurement reportedly measures only soluble sulfides (Cline, 1969), but the reagent used was very acidic and could have dissolved some of the acid-soluble sulfide in FeS precipitate. Therefore, the values reported for sulfide can not be used in a sulfur mass balance, but serve to confirm sulfide production qualitatively.

The rates of degradation ranged from 0.1 to 1.5 mg L<sup>-1</sup> day<sup>-1</sup> depending on the substrate mixture, substrate concentration, and culture conditions. Typically, toluene and *m*-xylene were degraded more rapidly than *p*- and *o*-xylene. The degradation of toluene, *p*-, and *o*-xylene in a mixture is shown in Figure 30. The degradation appears to be sequential, with toluene being degraded the first, and *o*-xylene the last. In the presence of more easily degradable substrates such as lactate, glucose, or yeast extract, the degradation of aromatic hydrocarbons ceased completely until the preferential substrates were consumed. This suggests that the degradation of aromatic hydrocarbons in the field may be slowed down or completely precluded even if the conditions are optimal and the appropriate physiological groups of microorganisms present, due to preferential degradation of more easily biodegradable substrates.

At total concentrations of toluene and xylenes above about 0.3 mM, the rates of degradation began to decrease. For example, at 0.4 mM, the rate of *m*-xylene degradation was 60% of the rate at 0.2 mM; at 0.6 mM, no degradation was observed. The optimum pH for degradation was found to be near 7.0 for both toluene and xylenes. At pH 6.0, the rate of toluene degradation was 80% of the rate at pH 7.0, and at pH 8.0, the rate of toluene degradation was 60% of the rate at pH 7.0. Free sulfide strongly inhibited degradation. The addition of 1 mM Na<sub>2</sub>S decreased the rates of toluene and xylene degradation by half, whereas 5 mM Na<sub>2</sub>S almost completely inhibited degradation. The rates of degradation would increase again if the cultures were flushed with N<sub>2</sub>/CO<sub>2</sub> and replenished with fresh medium, or if they were amended with 2 mM FeSO<sub>4</sub> to precipitate free sulfide from solution.

<sup>14</sup>C-labeling studies (with either [<sup>14</sup>C]ring-labeled toluene, [<sup>14</sup>C]methyl-labeled toluene, or [<sup>14</sup>C]methyl-labeled *o*-xylene) indicated complete mineralization of aromatic hydrocarbons. 95-100% of the initial volatile activity was recovered as <sup>14</sup>CO<sub>2</sub> in all cases, whereas only a very small portion of the carbon was assimilated

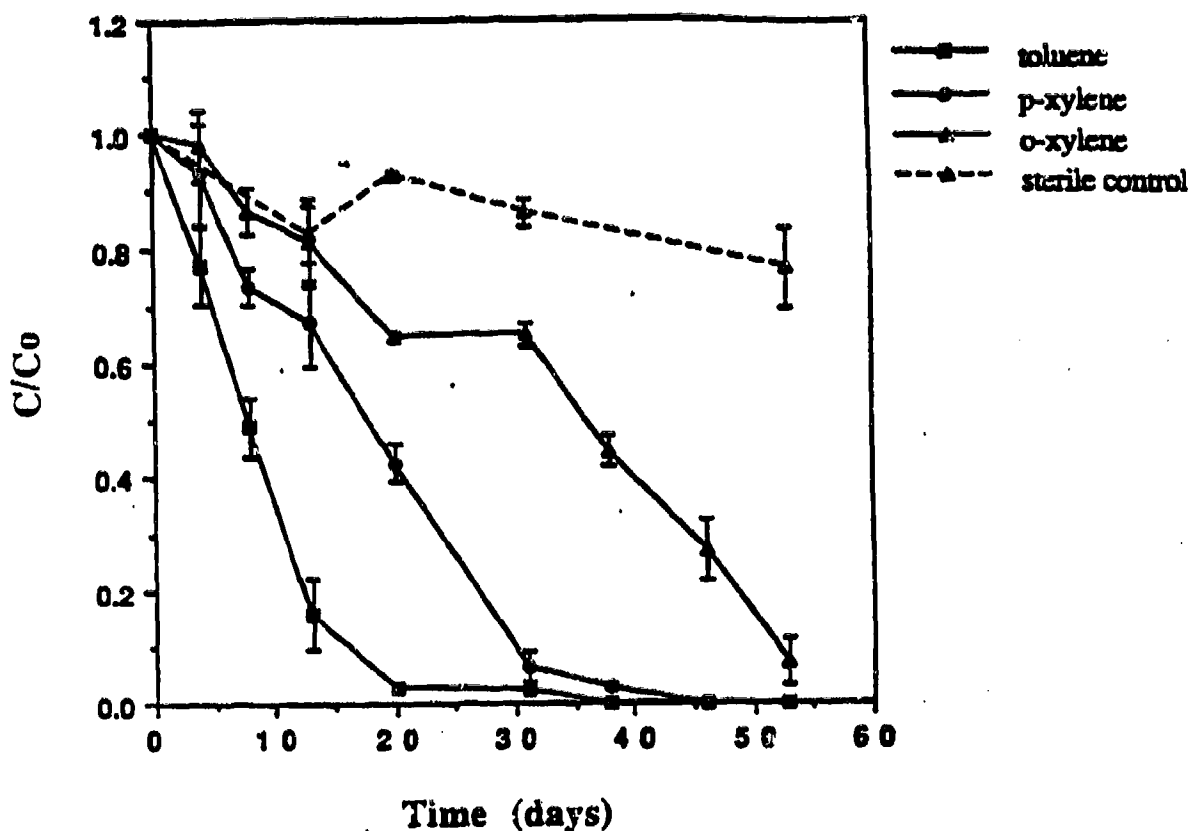


Figure 30. Sequential degradation of toluene, *p*-xylene, and *o*-xylene by a sulfate-reducing mixed culture. The compounds were fed simultaneously at an initial concentration of each compound ranging from 0.8 to 0.12 mM. The results for active enrichments are the mean of three replicates. The results for the sterile control are the mean of two replicates for all three substrates. Error bars are ( $\pm$ ) standard deviation



into cells (Figure 31). The cell counts also indicated that the cultures exhibited a small cell yield (total cell counts ranged from  $10^6$  cells  $\text{mL}^{-1}$  to  $5 \times 10^7$  cells  $\text{mL}^{-1}$ ) and very long doubling times, as would be expected under sulfate-reducing conditions (Table 7). However, the measured yields were lower than the theoretically calculated yields; this may be due to suboptimal incubation conditions (presence of free sulfide that acts as an inhibitor). Under epifluorescence microscopy, all the cells in the community appeared in close association with solid particles and with each other, which underscores the importance of attachment for the interspecies transfer of intermediates and products.

Although benzene was not degraded in the hydrocarbon mixture, it was completely mineralized when used as sole substrate. This indicates that the recalcitrance of benzene under anaerobic conditions, repeatedly observed in the field (Wilson et al., 1986, 1987; Major et al., 1988), may not be as much the result of benzene structure or the absence of capable organisms, but rather due to the phenomenon of preferential substrate utilization. Benzene was fed to six replicate microcosms in liquid concentrations ranging from 0.04 to 0.2 mM. One of the microcosms also received 0.14 mM toluene as a positive control for the presence of active aromatic hydrocarbon degraders. Toluene in this microcosm was completely degraded in less than 30 days. Benzene degradation began in all the active microcosms after at least a 30-day lag time (Table 8). The initial rate of benzene degradation increased as initial concentrations increased up to 0.14 mM. However, at an initial concentration of 0.2 mM, a longer lag time and much slower rate of degradation were observed, probably as a result of substrate toxicity. The rate of benzene degradation in microcosm #3, which was also amended with toluene, was considerably slower than the rate in a parallel microcosm (# 2) that did not receive toluene. Subsequent refeeding of benzene to the microcosms resulted in degradation without a lag (Figure 32).

$^{14}\text{C}$ -studies showed that about 90% of the label from benzene was converted to  $^{14}\text{CO}_2$ . No  $^{14}\text{CO}_2$  was detected in the sterile microcosm (Figure 33). Although no conclusive connection between benzene degradation and sulfate reduction was established, some observations indicate that sulfate was used as an electron acceptor in this process. First, no nitrate was present in the sediment or in the medium, eliminating the possibility of denitrification. Second, a negligible amount of methane was produced in each microcosm ( $0.3\% \pm 0.1\%$  in the headspace), which would be

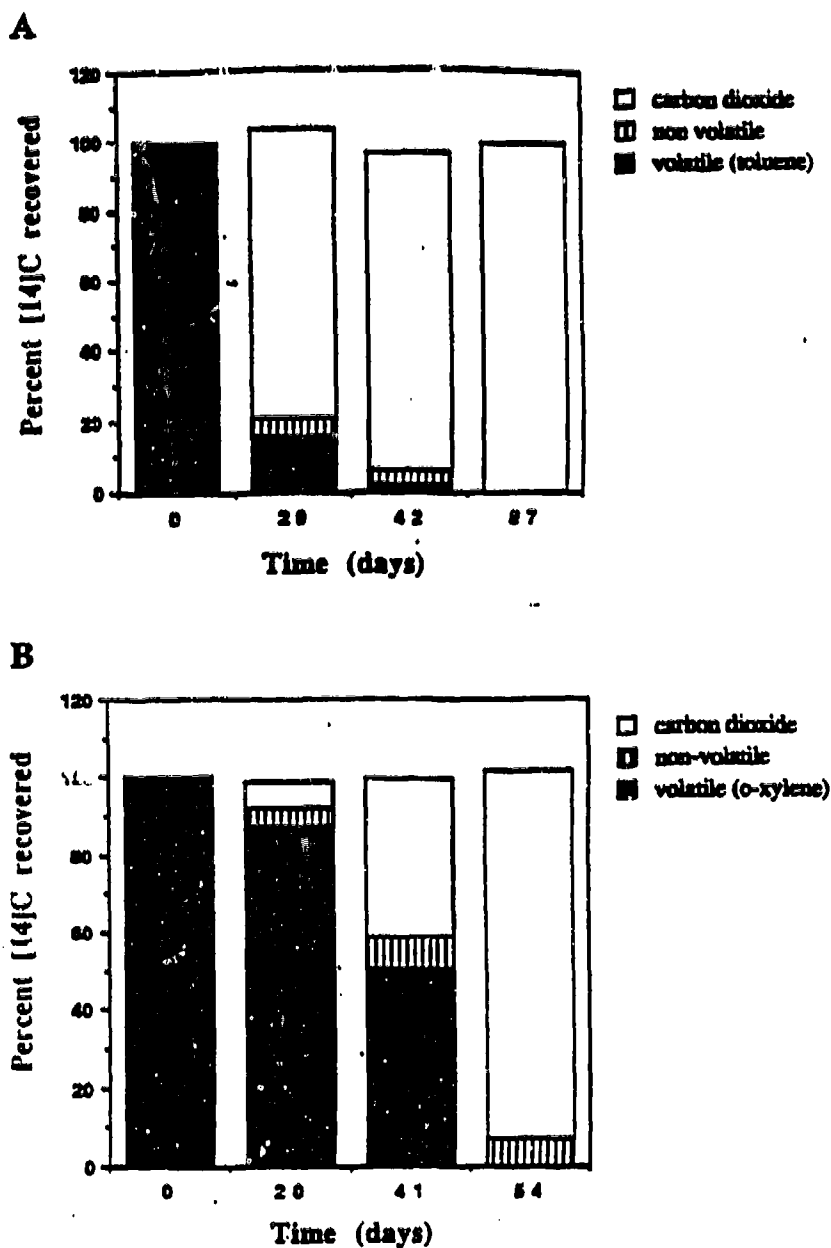


Figure 31. Degradation of  $[^{14}\text{C}]$ -labeled substrates in sulfate-reducing mixed cultures. The data in panel A are the means from two cultures incubated with  $[^{14}\text{C}]$ methyl-labeled toluene. The data in panel B are the means from two cultures incubated with  $[^{14}\text{C}]$ methyl-labeled *o*-xylene. The nonvolatile fraction includes nonvolatile intermediates and biomass. No change in  $^{14}\text{C}$  distribution was observed in sterile controls

Table 7. Cell yield and doubling time for a sulfate-reducing mixed culture growing on toluene and *m*-xylene

Growth Substrate	Yield <sup>a,b</sup> (g Cells/ g Substrate)	Doubling Time <sup>a</sup> (Days)
Toluene	0.10 ± 0.02	22 ± 4.4
<i>m</i> -Xylene	0.14 ± 0.03	20 ± 4.0

<sup>a</sup> Values are mean ± standard deviation of 2 or 3 replicates.

<sup>b</sup> The calculated theoretical maximum cell yield for growth on toluene or *m*-xylene is about 0.2 g cells (dry weight)/ g substrate (McCarty, 1971).

Table 8. Lag times before degradation and initial rates of degradation of benzene in anaerobic microcosms

Microcosm #	Initial Benzene Concentration (uM)	Approximate Lag Time (Days)	Initial Rate <sup>a</sup> (uM day <sup>-1</sup> )
1	40	30-60	1.0
2	90	30-60	1.9
3(+ toluene) <sup>b</sup>	90	30-60	0.8
4	140	30-60	3.7
5	200	70-100	0.4
6 (sterile)	90	no degradation	

<sup>a</sup> The initial rate was defined as the rate for the first feeding (i.e. after the onset of degradation).

<sup>b</sup> Toluene (140 uM) was added as a positive control for degradation and was completely degraded in less than 30 days.

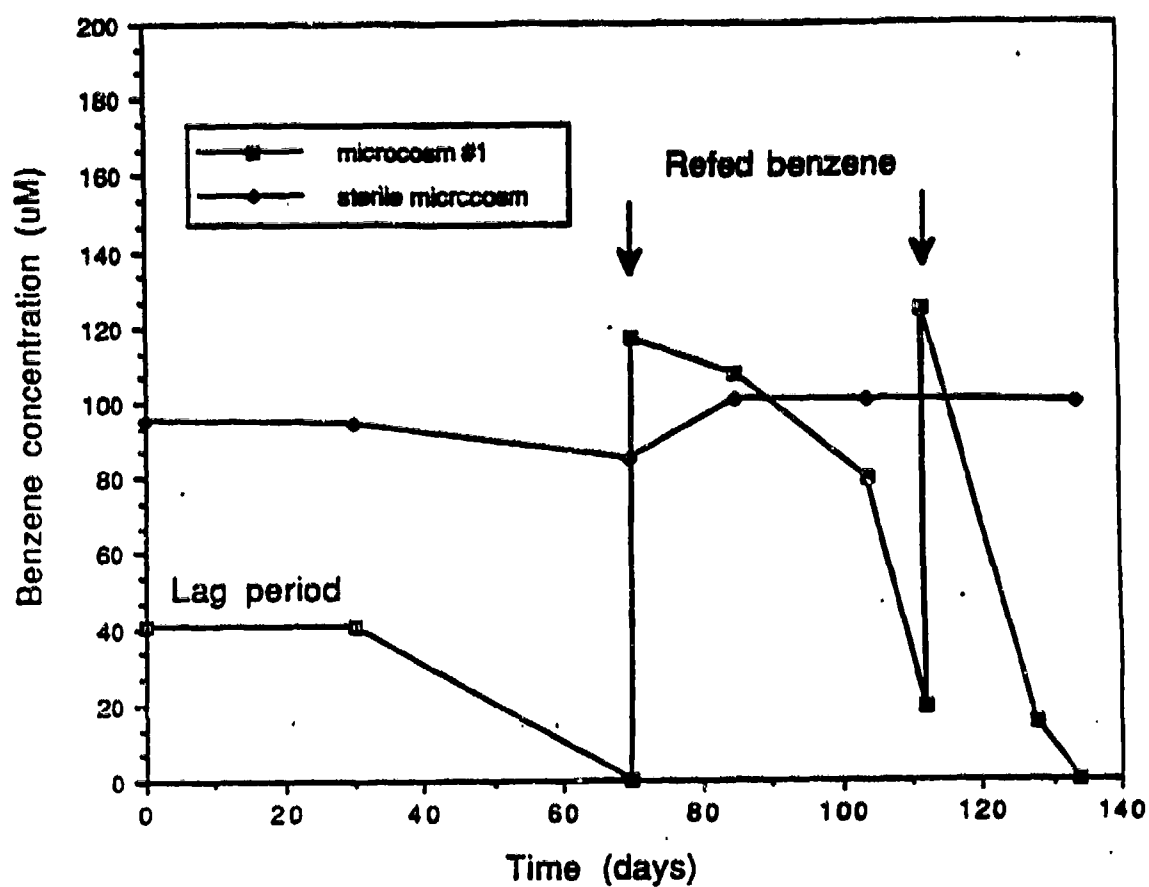


Figure 32. Benzene degradation in a typical active microcosm compared to sterile control. The active microcosm was refed benzene on the days indicated by arrows

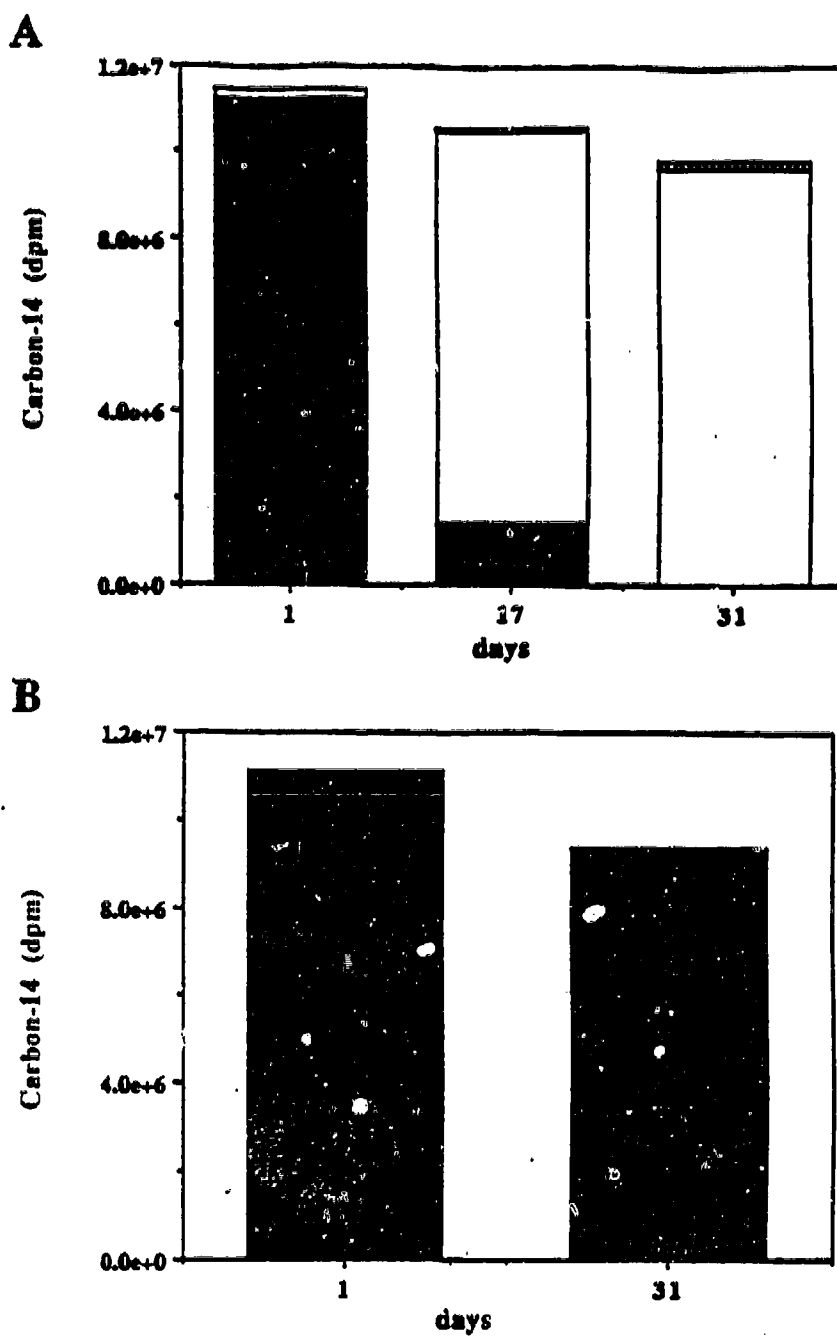


Figure 33. Degradation of [ $^{14}\text{C}$ ]benzene in anaerobic microcosms.

(A) Microcosm #1. (B) Sterile control (# 6). Solid bars, volatile fraction (benzene); hatched bars, nonvolatile fraction; open bars,  $\text{CO}_2$ .

equivalent to only 5% of the benzene actually degraded. Third, the amount of  $\text{CO}_2$  produced was more consistent with sulfate reduction than methanogenesis. Fourth, the sulfate concentration in active microcosms decreased slightly relative to the sterile control, from the initial 20.0 mM sulfate to  $19.5 \pm 0.3$  mM. This change in concentration corresponds to the theoretical amount of sulfate needed to degrade 140 mM of benzene, if all the sulfate were reduced to sulfide. However, the role of oxidized metals in the sediment, as well as of  $\text{CO}_2$ , can not be excluded; likewise, the possibility can not be excluded that sulfate was depleted as an electron acceptor for the oxidation of unknown electron donors present in the sediment.

## **CBs IN STABLE METHANOGENIC CONSORTIA**

Stable ferulate-degrading methanogenic cultures, originally enriched from anaerobic sludge, did not transform HCB, PeCB, TTCB, or TCB. However, they did support slow transformation of MCB and of two isomers of DCB (1,4- and 1,3-DCB; 1,2-DCB was not transformed). Figure 34 shows MCB transformation by ferulate-fed methanogenic consortia. After 91 days of incubation at 35°C, active cultures removed an average of 29% of the initial MCB concentration (20 mg L<sup>-1</sup>) relative to sterile chemical and autoclaved biological controls. The loss of MCB (and other CBs tested) in the controls may be ascribed to volatilization and dissolution in the teflon-coated valves that were used to stopper the bottles. Upon refeeding of MCB and ferulate, an average of 14% of MCB was removed during 72 days of incubation, which represents an increase in rate (about 81 ug of MCB day<sup>-1</sup>) when compared to the transformation after the first feeding (about 66 ug of MCB day<sup>-1</sup>). This would indicate an acclimation of the ferulate consortia to MCB transformation. Ferulate was completely degraded as indicated by stoichiometric production of CO<sub>2</sub> and CH<sub>4</sub>. The transformation of MCB in the presence of ferulate could have been either a cometabolic process, or secondary substrate utilization, or utilization of MCB as an electron acceptor, with the electrons being derived from ferulate. To address this question, <sup>14</sup>C-MCB studies were performed. Three ferulate-degrading consortia were treated as before (addition of ferulate plus <sup>14</sup>C-MCB) whereas one consortium was amended with <sup>14</sup>C-MCB only (no ferulate) after the degradation of ferulate had been completed. After 132 days of incubation, the ferulate-amended consortia converted up to 6% of the <sup>14</sup>C-MCB to aqueous (nonvolatile) intermediates, and up to 1.2% to <sup>14</sup>CO<sub>2</sub>. The consortium fed MCB without ferulate converted 20% of the MCB to nonvolatile intermediates, and 7.5% to <sup>14</sup>CO<sub>2</sub>. This suggests that ferulate was actually slowing down the MCB utilization process, and that the methanogenic consortia could slowly utilize MCB as a primary substrate (electron donor, carbon source, and/or energy source) in the absence of ferulate. The effect of ferulate can be explained by the fact that the consortium had been originally adapted to this compound as the primary substrate, and by the high ferulate concentration (5 mM, i.e. 50 mM carbon) relatively to MCB concentration (about 0.2 mM, i.e. only 1.2 mM carbon). The pathway of MCB degradation remains unknown; no intermediates were detected using the described GC/MS procedure. However, the fact that a small amount of <sup>14</sup>CO<sub>2</sub> was produced from <sup>14</sup>C-MCB indicates that the aromatic ring must have been broken, which would require an initial activation



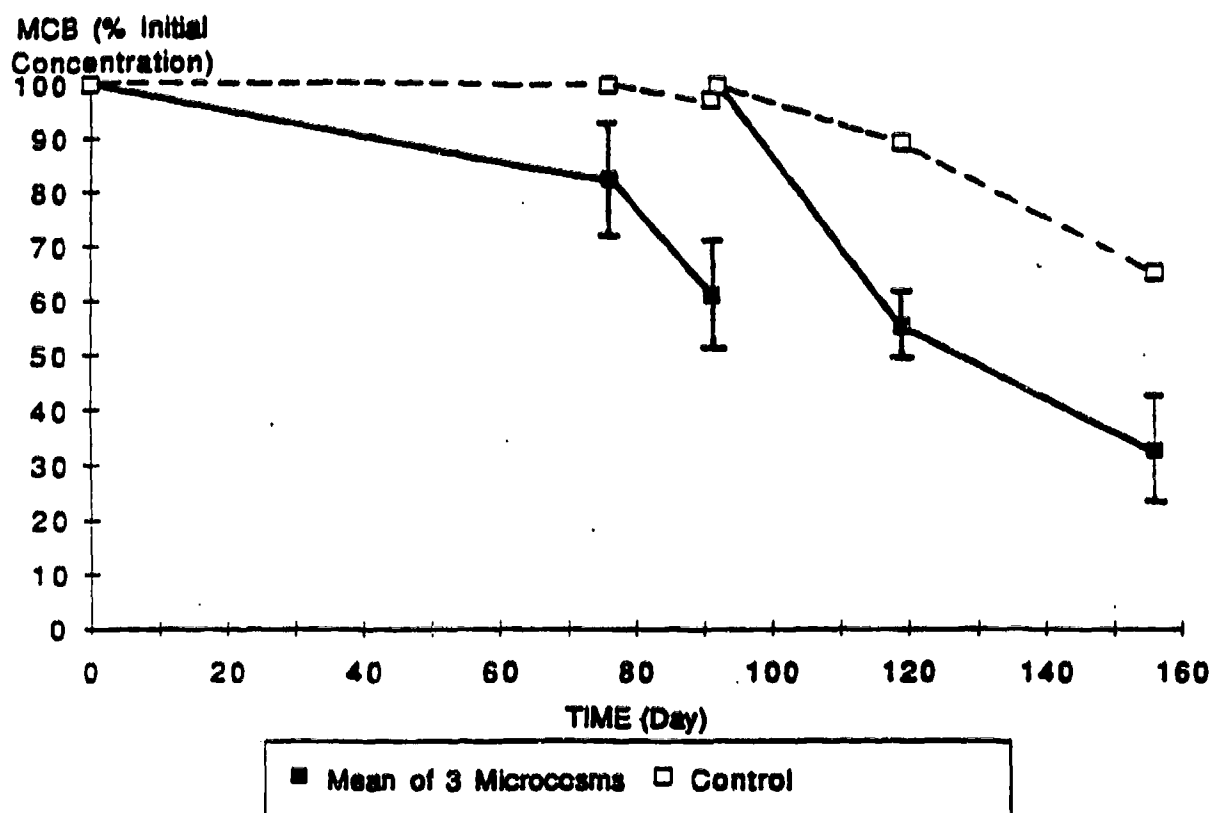


Figure 34. MCB transformation in ferulate-degrading methanogenic consortia

(e.g., an oxidation with the oxygen derived from water, or some other, unknown mechanism).

The ferulate consortia also transformed 1,4-DCB (Figure 35) and 1,3-DCB. 1,4-DCB was spiked to the cultures at an initial concentration of 20-50 mg L<sup>-1</sup>, and 50% was removed (relative to the chemical and autoclaved controls) in 90 days of incubation at 35°C, in the presence of ferulate as the primary substrate. After 30 days of incubation, MCB was found as a transformation product (0.15 mM MCB formed per 1 mM 1,4-DCB transformed), indicating reductive dechlorination of DCB. <sup>14</sup>C-DCB studies indicated that nonvolatile aqueous intermediates were also produced (up to 85% of the initial 1,4-DCB concentration in 14 days of incubation); this process was accompanied by MCB disappearance. The appearance of nonvolatile intermediates was transient, but no <sup>14</sup>CO<sub>2</sub> was detected. The other DCB isomer, 1,3-DCB, was also transformed (67.3% of the initial 20-50 mg L<sup>-1</sup> removed during 90 days of incubation). No <sup>14</sup>C-labeling studies were performed, and no intermediates of transformation were detected using several different chromatographic procedures (GC, GC/MS, HPLC).

In summary, methanogenic ferulate-degrading consortia can transform CB with a low degree of chlorination (MCB, DCB), but not higher chlorinated CBs; this is possibly due to the lack of the enzymatic capacity for these large molecules with bulky chlorine substituents. DCB seem to be reductively dechlorinated, and it is likely that the electrons for this process are derived from ferulate as an electron donor, since there was no other substrate in the medium. MCB, however, is transformed through a different process which is possibly hydrolytic and results in a small amount of MCB being completely mineralized to CO<sub>2</sub>. All the transformations are slow (on the order of months), but might be significant in nature, in complex methanogenic communities that feed on plant-derived aromatic compounds.

## **CBs IN AQUIFER MICROCOSMS**

### **A. MCB**

MCB was transformed only in the microcosms containing methanogenic material from the Pensacola (FL) creosote-contaminated aquifer. These microcosms were amended with 0.25 mM MCB dissolved in 0.1 mL methanol per 100-mL microcosm

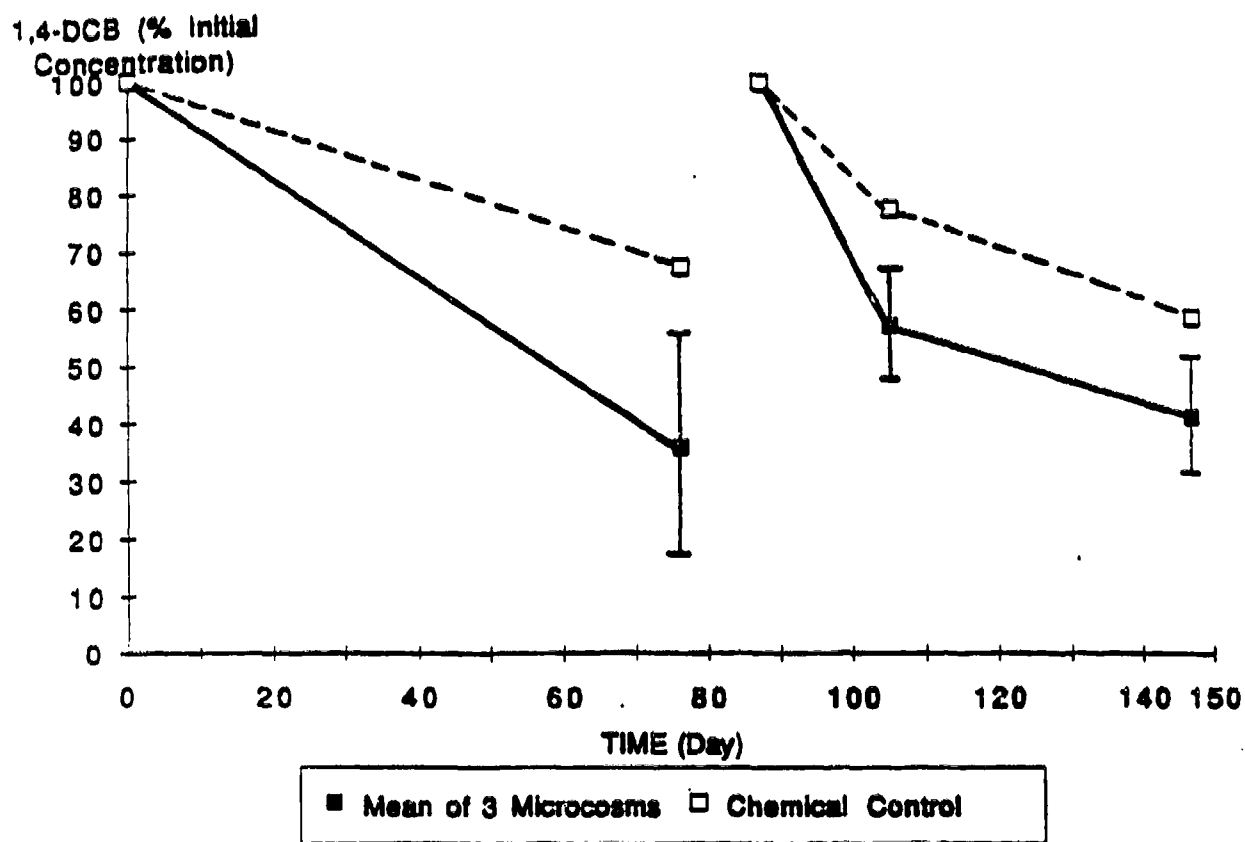


Figure 35. 1,4-DCB transformation by ferulate-fed methanogenic consortia

volume (aquifer solids plus liquid medium). The results are shown in Figure 36. The disappearance of MCB occurred in active microcosms as well as sterile controls, but it was faster in the active microcosms. In the absence of microorganisms, the removal of MCB from the liquid phase occurred most likely due to sorption to aquifer solids and to the stopper valves. In the presence of microorganisms, this removal was enhanced by biological uptake and transformation; a faster rate of removal upon refeeding indicates adaptation of the microorganisms to MCB. Furthermore, the active microcosms were consistently producing a small amount of  $\text{CO}_2$  and  $\text{CH}_4$ , whereas no gases were produced by the controls. After the second refeeding of MCB, however, MCB removal stopped, suggesting that some essential nutrients or electron donors from the aquifer material were used up. To find an answer to this question, three microcosms were spiked with  $^{14}\text{C}$ -MCB; one of them (microcosm A) was amended with  $123 \text{ mg L}^{-1}$  acetate as a carbon source and electron donor, microcosm B was amended with  $22 \text{ mg L}^{-1}$  benzoate, and microcosm C with  $156 \text{ mg L}^{-1}$  benzoate. As shown in Table 9, a portion of  $^{14}\text{C}$ -MCB was converted to water-soluble intermediates, and a small portion to  $\text{CO}_2$ ; however, all of the chromatographic methods used (GC, HPLC, GC/MS) failed to detect any intermediates. The acetate-amended microcosm A transformed MCB the fastest; microcosm B, with a low concentration of benzoate, was slower; and microcosm C, with a high concentration of benzoate, the slowest. Benzoate, on the other hand, was completely degraded in microcosm B in 20 days, and in the microcosm C, in about 70 days. A possible interpretation of these results is that MCB is degraded by the anaerobic community that degrades aromatic compounds; in the presence of a preferential aromatic substrate (benzoate), the MCB transformation is delayed. It is not likely that MCB is reductively dechlorinated to benzene, because aqueous intermediates (which must be oxidized compounds) are produced, as well as  $\text{CO}_2$ . However, a possibility of MCB cometabolism in the presence of acetate or some other organic compound on the aquifer solids, can not be excluded.

## B. Higher Chlorinated CBs

Freshly sampled Pensacola aquifer material was preincubated without any additions in an anaerobic chamber at  $35^\circ\text{C}$  for four days; after this period, the microcosms were spiked with methanol solutions of 1,4-DCB, 1,2,3-TCB, 1,2,3,5-TTCB, PeCB, and HCB as single substrates, in concentrations from 150 to  $1,000 \text{ ug L}^{-1}$ . During a 119-day incubation period, no transformation of 1,4-DCB occurred, whereas

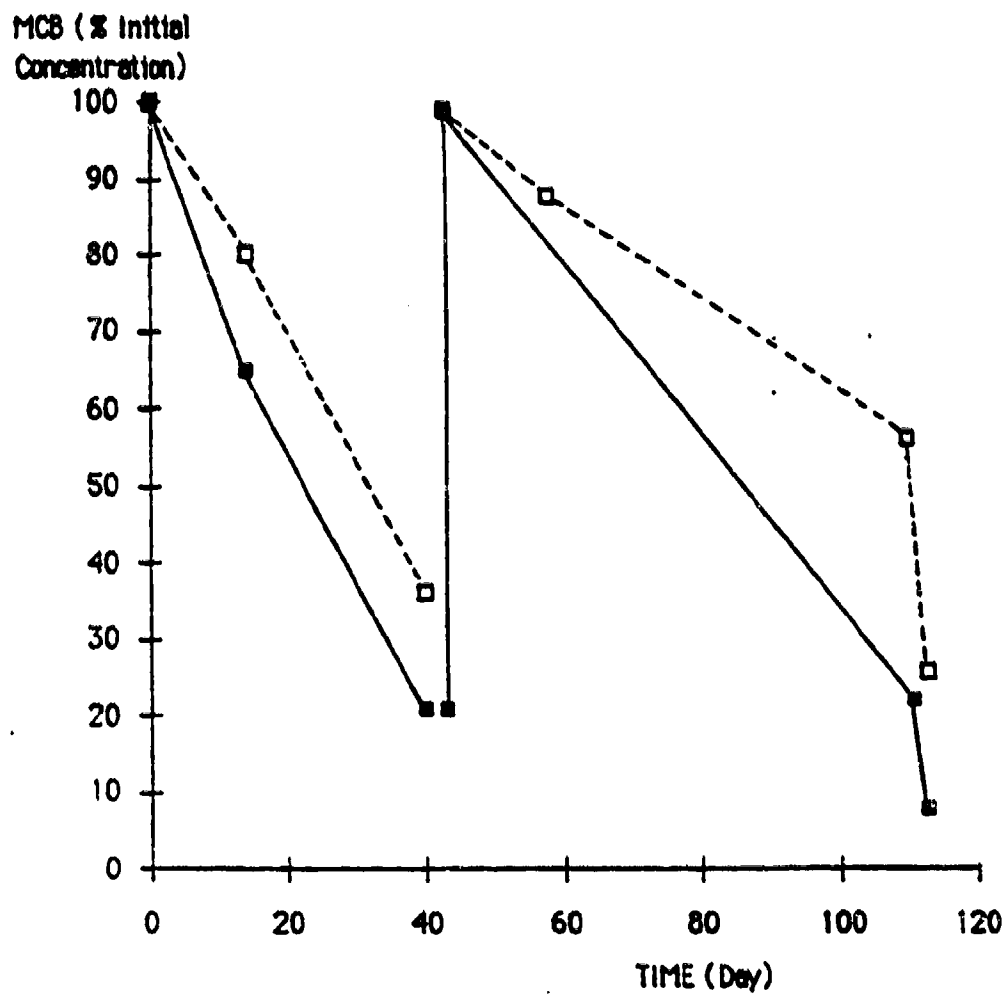


Figure 36. Anaerobic transformation of monochlorobenzene in methanogenic microcosms derived from the Pensacola aquifer (solid line). The dashed line represents the sterile control. The substrate was refed on days 43 and 118 to both active microcosms and the controls

Table 9. Percentage of the total  $^{14}\text{C}$ -MCB converted to nonvolatile aqueous intermediates (AQ) and  $\text{CO}_2$  by methanogenic consortia derived from the Pensacola aquifer during 132 days of incubation. The results have been corrected for background values found in triplicate controls

Incubation Time	Microcosm A	Microcosm B	Microcosm C
0 Days	AQ 0%	AQ 0%	AQ 0%
	$\text{CO}_2$ 0%	$\text{CO}_2$ 0%	$\text{CO}_2$ 0%
14 Days	AQ 18%	AQ 3%	AQ 3%
	$\text{CO}_2$ 0%	$\text{CO}_2$ 0%	$\text{CO}_2$ 0%
31 Days	AQ 16%	AQ 7%	AQ 4%
	$\text{CO}_2$ 4%	$\text{CO}_2$ 2%	$\text{CO}_2$ 0.5%
132 Days	AQ 15%	AQ 10%	AQ 6%
	$\text{CO}_2$ 3%	$\text{CO}_2$ 6%	$\text{CO}_2$ 1%

HCB was transformed both biotically and abiotically (in sterilized controls) without a significant difference in rates. The other compounds were reductively dechlorinated by the aquifer microflora as described in the following.

1,2,3-TCB was reductively dechlorinated to 1,3-DCB as determined by GC and GC/MS. The transformation started without a lag. The amount of 1,3-DCB produced was stoichiometrically very close to the amount of 1,2,3-TCB added. No 1,2-DCB or 1,4-DCB were detected; trace concentration (0.69  $\mu\text{M}$ ) of MCB was found later in incubation (after 88 days). The initial rate of transformation (during the first 37 days of incubation) was on the order of 68 nmol of 1,2,3-TCB  $\text{L}^{-1} \text{d}^{-1}$ , but subsequently decreased (between days 48 and 60) to 34 nmol  $\text{L}^{-1} \text{d}^{-1}$ . However, upon the refeeding of 1,2,3-TCB, the rate increased substantially to 1,068 nmol  $\text{L}^{-1} \text{d}^{-1}$  as measured during a ten-day period after refeeding; this indicated acclimation of the microflora to the TCB. It needs to be pointed out that sterilized controls produced trace concentrations of MCB and 1,3-DCB (an order of magnitude less than in the active microcosms) from 1,2,3-TCB late in incubation (over a hundred days), suggesting that abiotic processes, possibly catalyzed by thermostable microbial products, may be occurring in the contaminated samples.

The active 1,2,3-TCB dechlorinating microcosms were used as a source for enrichment of partially suspended mixed cultures that still contained a small amount of solids. These enrichments were obtained by transferring 20 mL of the microcosm fluid into 105 mL of the prereduced defined mineral medium. The enrichments continued to reductively dechlorinate 1,2,3-TCB added to the cultures as methanol solution; the transformation continued at rates comparable to the rates in microcosms (Figure 37). The initial concentration of 1,2,3-TCB measured and shown in the figure was lower than the amount added, due to sorption to solids and/or stoppers, as confirmed by measurements in sterilized controls. The amount of 1,3-DCB produced was stoichiometrically very close to the amount of 1,2,3-TCB added. Neither 1,2- nor 1,4-DCB were formed; MCB was found in small concentrations (up to 0.96  $\mu\text{M}$ ).

1,2,3,5-TTCB was reductively dechlorinated almost stoichiometrically to 1,3,5-TCB as the major product; however, formation of trace levels (0.4  $\mu\text{M}$  or less) of 1,3-DCB and MCB was also detected during later stages of incubation. The dechlorination started without a lag. Sterilized controls produced some 1,3,5-TCB, but the concentration was two orders of magnitude lower than in the active microcosms.

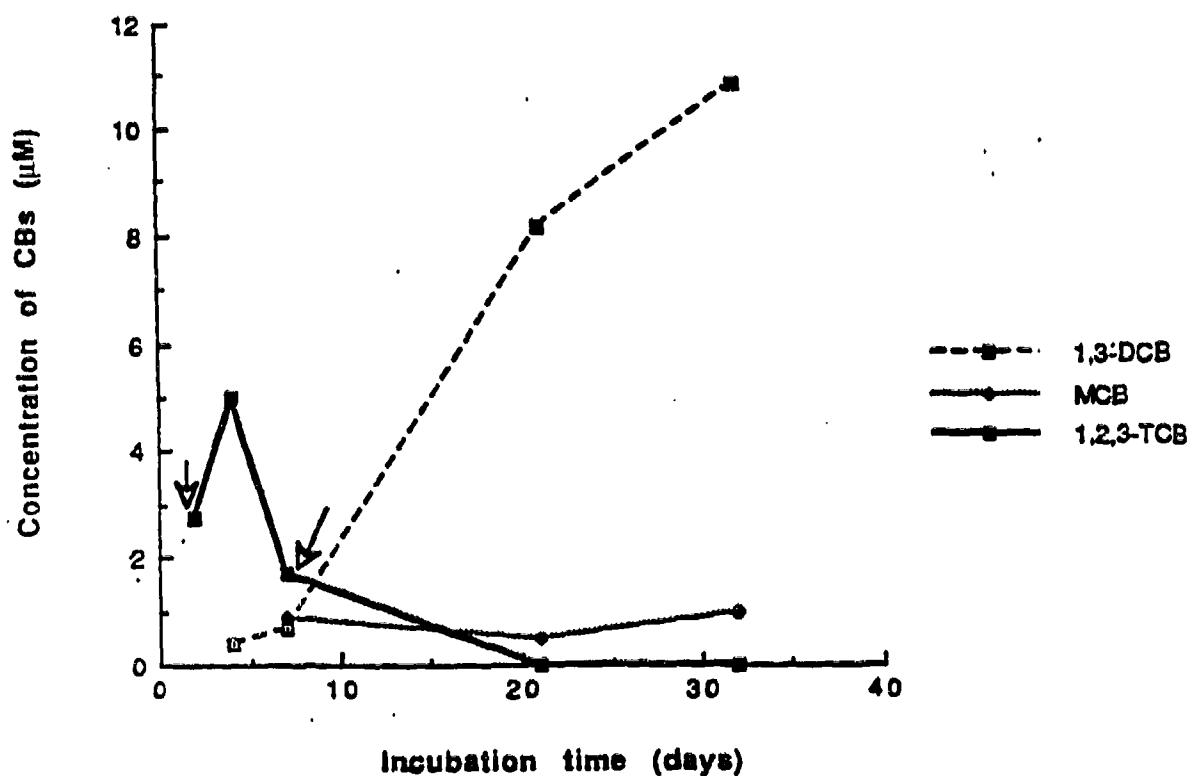


Figure 37. Reductive dechlorination of 1,2,3-TCB to 1,3-DCB and MCB in enrichments derived from microcosms with the anaerobic Pensacola aquifer material, without the addition of accessory electron donors. The results obtained from triplicate cultures are corrected for the background values measured in duplicate controls. The arrows indicate the days on which 1,2,3-TCB was respiked



Similarly to the 1,2,3-TCB microcosms, the rates were greatly enhanced upon acclimation: initially they were on the order of 435 nmol of 1,2,3,5-TTCB L<sup>-1</sup> d<sup>-1</sup>, which was increased upon respiking of 1,2,3,5-TTCB to 1,459 nmol L<sup>-1</sup> d<sup>-1</sup>. Enrichments obtained from the microcosms transformed the TTCB at rates only slightly lower than the microcosms (Figure 38), with 1,3,5-TCB as the major and 1,3-DCB as a minor product. Traces of MCB (0.3 µM or less) were also found (not shown in the figure).

PeCB was reductively dechlorinated, without a lag, to 1,2,3,5-TTCB, 1,3,5-TCB, and traces (approximately 0.2 µM or less) of 1,3-DCB and MCB during the later stages of transformation. 1,3,5-TCB was the major product. Sterilized controls also produced 1,2,3,5-TTCB and 1,3,5-TCB, but in concentrations two orders of magnitude lower. The rate of PeCB transformation by the active microcosms during the first 50 days was on the order of 65 nmol L<sup>-1</sup> d<sup>-1</sup>, to be increased upon respiking to 123 nmol L<sup>-1</sup> d<sup>-1</sup>. The enrichments obtained from the active microcosms exhibited an identical dechlorination pattern, but the rates were significantly slower than in the microcosms (about 12 nmol of PeCB L<sup>-1</sup> d<sup>-1</sup> between days 30 and 90; 44 nmol L<sup>-1</sup> d<sup>-1</sup> upon respiking). 1,3,5-TCB was again the major product (Figure 39), whereas 1,3-DCB and MCB were produced in trace amounts (at or below 0.6 µM and 0.4 µM, respectively). It is interesting to note the repeating dechlorination pattern in the 1,2,3-TCB, 1,2,3,5-TTCB, and PeCB-dechlorinating microcosms and cultures: the chlorine which is situated between two other chlorines is preferentially removed (resulting in production of 1,2,3,5-TTCB and 1,3,5-TCB from PeCB; 1,3,5-TCB from 1,2,3,5-TTCB; and 1,3-DCB from 1,2,3-TCB). Once there are no adjacent chlorine substituents left, the rate of dechlorination ceases: only traces of 1,3-DCB are produced from 1,3,5-TCB, whereas, on the contrary, 1,2,3-TCB is quickly and stoichiometrically reduced to 1,3-DCB. As previously suggested by Alexander and Lustigman (1966), *meta*-substituted benzenes are more stable than *ortho*-substituted ones. Our results correspond to the results of Fathepure et al. (1988), obtained with anaerobic sludge communities dechlorinating HCB; this is significant, because it indicates that similar transformation reactions can be expected for CBs under anaerobic conditions regardless of the inoculum source.

Microscopic examination of the enrichments dechlorinating TCB, TTCB, and PeCB revealed at least three different morphological types in all the enrichments: straight rods, 0.3 x 1 µm; slightly larger rods, 0.4 x 1 µm; and slightly curved rods, 0.2 x 0.5 µm. The attempts to isolate pure cultures were unsuccessful. The addition of 2 mM BESA to the TTCB- and PeCB-dechlorinating enrichments decreased the rate of

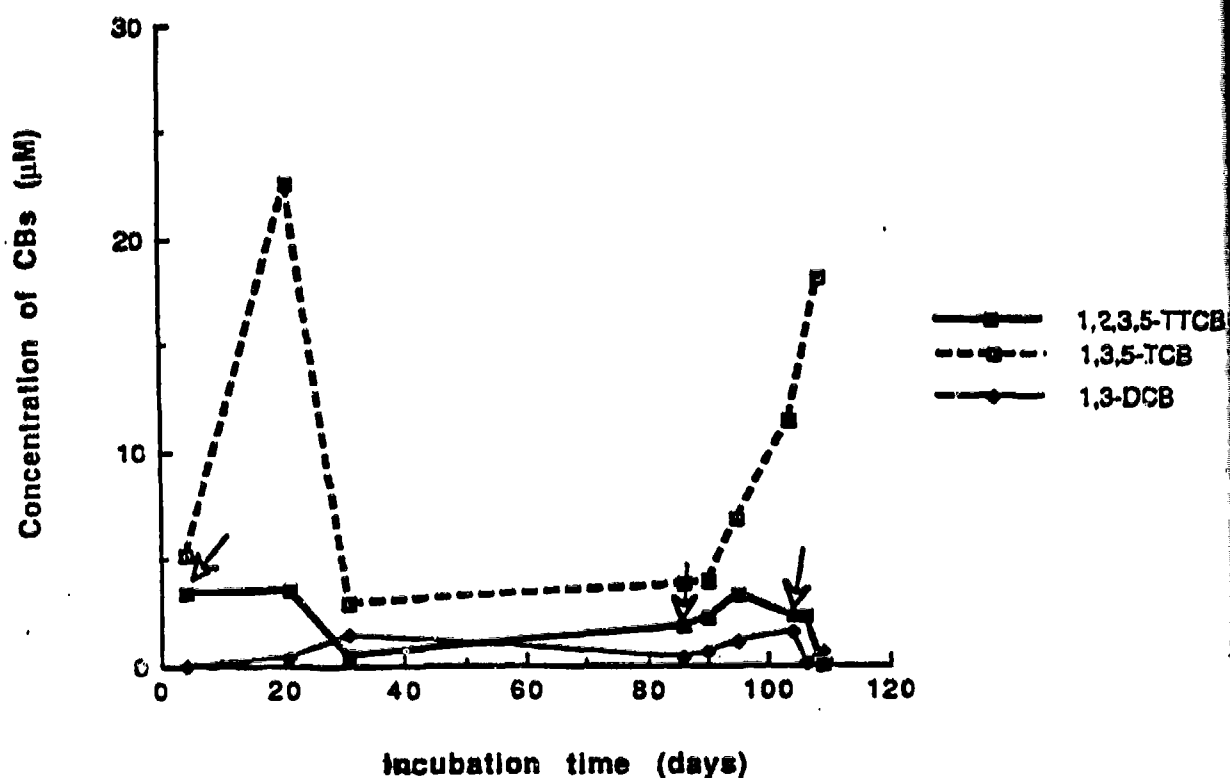


Figure 38. Reductive dechlorination of 1,2,3,5-TTCB to 1,3,5-TCB and 1,3-DCB in enrichments derived from microcosms with the anaerobic Pensacola aquifer material, without the addition of accessory electron donors. The initial concentration of 1,2,3,5-TTCB measured and shown in the figure was lower than the amount added, due to sorption to solids and/or stoppers, as confirmed by measurements in sterilized controls. The amount of 1,3,5-TCB produced was stoichiometrically very close to the amount of 1,2,3,5-TTCB added. The results obtained from triplicate cultures are corrected for the background values measured in duplicate controls. The arrows indicate the days on which 1,2,3,5-TTCB was respiked

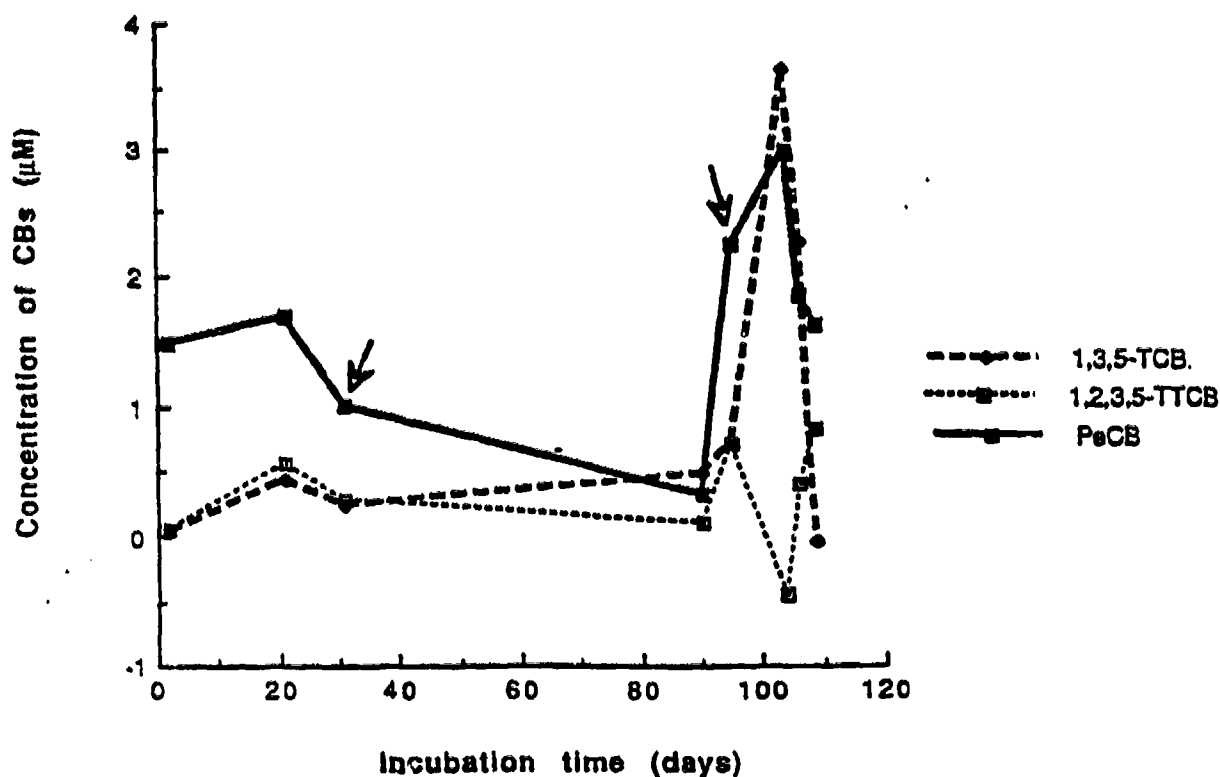


Figure 39. Reductive dechlorination of PeCB to 1,2,3,5-TTCB and 1,3,5-TCB in enrichments derived from microcosms with the anaerobic Pensacola aquifer material, without the addition of accessory electron donors. The initial concentration of PeCB measured and shown in the figure was lower than the amount added, due to sorption to solids and/or stoppers, as confirmed by measurements in sterilized controls. The amount of 1,3,5-TCB produced was stoichiometrically very close to the amount of PeCB added. The results obtained from triplicate cultures are corrected for the background values measured in duplicate controls. The arrows indicate the days on which PeCB was respiked.

reductive dechlorination and the rate of 1,3,5-TCB production, indicating that a methanogenic community was involved in the dechlorination process. The results for TTCB are shown in Table 10.

The 1,2,3-TCB, 1,2,3,5-TTCB, and PeCB enrichment cells were harvested and washed and used in cross-acclimation studies, in which each type of the cells was resuspended in fresh prereduced defined medium and amended with the other two CBs as sole substrates. The 1,2,3-TCB enrichment cells were able to reductively dechlorinate 1,2,3,5-TTCB, but not PeCB. It is interesting to note that the TTCB was transformed faster than the TCB: during 20 days of incubation, 60% of the initial maximum concentration of TTCB (8  $\mu$ M) was dechlorinated, while in the same period only 44% of the initial maximum TCB concentration (7  $\mu$ M) was dechlorinated. Under the assumption that similar dechlorinating microbial communities are enriched by both TCB and TTCB, this result is to be expected, because highly chlorinated CBs are more prone to reductive dehalogenation than the lesser chlorinated ones (Fathepure et al., 1988). The 1,2,3,5-TTCB enrichment cells transformed the TTCB and TCB with similar efficiencies, but they were also capable of dechlorinating PeCB with an efficiency significantly higher than either TTCB or TCB (Figure 40). The results obtained with the PeCB enrichment cells were somewhat surprising: these cells transformed all of the compounds tested (PeCB, 1,2,3,5-TTCB, and 1,2,3-TCB), but PeCB was dechlorinated at a rate significantly slower than either TTCB or TCB (Figure 41). This may have been due to the high initial concentration of PeCB (16  $\mu$ M) which might have been inhibitory to the cells. Furthermore, significant abiotic dehalogenation of PeCB (24% of the initial maximum concentration of 24  $\mu$ M) was observed in autoclaved controls. The conclusion may be drawn that the microbial communities enriched on TCB, TTCB, and PeCB are not necessarily identical, and that highly chlorinated CBs can undergo a significant abiotic dehalogenation, possibly due to catalysis by thermostable microbial constituents (the dehalogenation was not observed in chemical controls that contained no autoclaved cells).

In an attempt to find out what were the potential electron donors for these reductive dechlorinations, a range of organics were tested. The addition of toluene did not increase the rate of dechlorination in the microcosms during the incubation period observed, which was probably to be expected considering the long lag period before the onset of toluene degradation in similar microcosms derived from Pensacola material (see the section "Methanogenic Degradation of MAH"). *p*-Cresol did not

Table 10. Rates of 1,2,3,5-TTCB transformation and 1,3,5-TCB production in the presence and absence of BESA in the anaerobic enrichment cultures derived from Pensacola microcosms<sup>a</sup>

CBs/Incubation Period (Days)	Rates (uM day <sup>-1</sup> )		
	Cultures Without BESA	Cultures With BESA	Autoclaved Controls
1,2,3,5-TTCB/ 0-5 days	-(0.42 ± 0.15) <sup>b</sup>	-(0.30 ± 0.10)	-(0.08 ± 0.18)
1,2,3,5-TTCB/ 52-55 days	-(0.64 ± 0.44)	-(0.08 ± 0.35)	-(0.02 ± 0.95)
1,3,5-TCB from TTCB/ 0-26 days	+(0.27 ± 0.09) <sup>c</sup>	+(0.10 ± 0.05)	0

<sup>a</sup> All the results are expressed as mean ± standard deviation of triplicate active cultures or duplicate autoclaved controls. Traces of MCB and 1,3-DCB were also produced in active cultures during transformation (data not shown).

<sup>b</sup> A negative sign means the compound concentration is declining (removal due to sorption and/or degradation).

<sup>c</sup> A positive sign indicates that the compound concentration is increasing (due to desorption and/or production from the parent substrate).

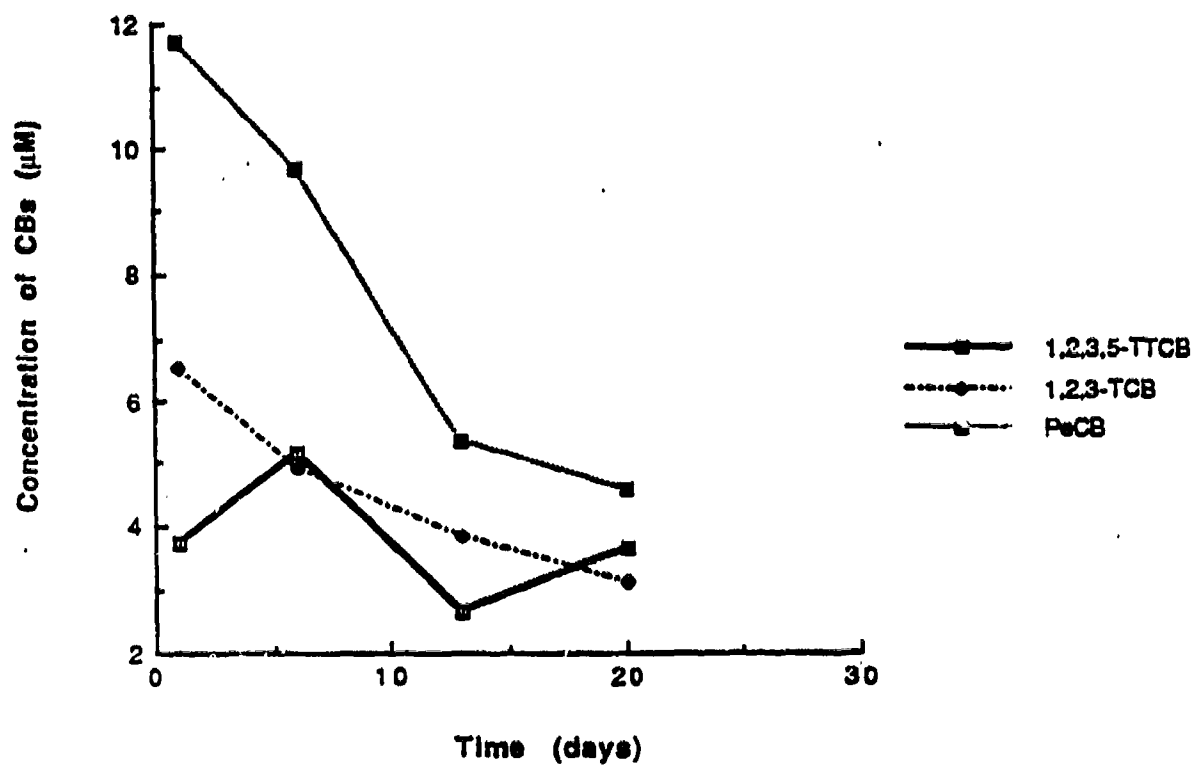


Figure 40. Reductive dechlorination of PeCB, 1,2,3,5-TTCB, and 1,2,3-TCB by the cells enriched on 1,2,3,5-TTCB. The results from triplicate cultures were corrected for the background values measured in duplicate controls

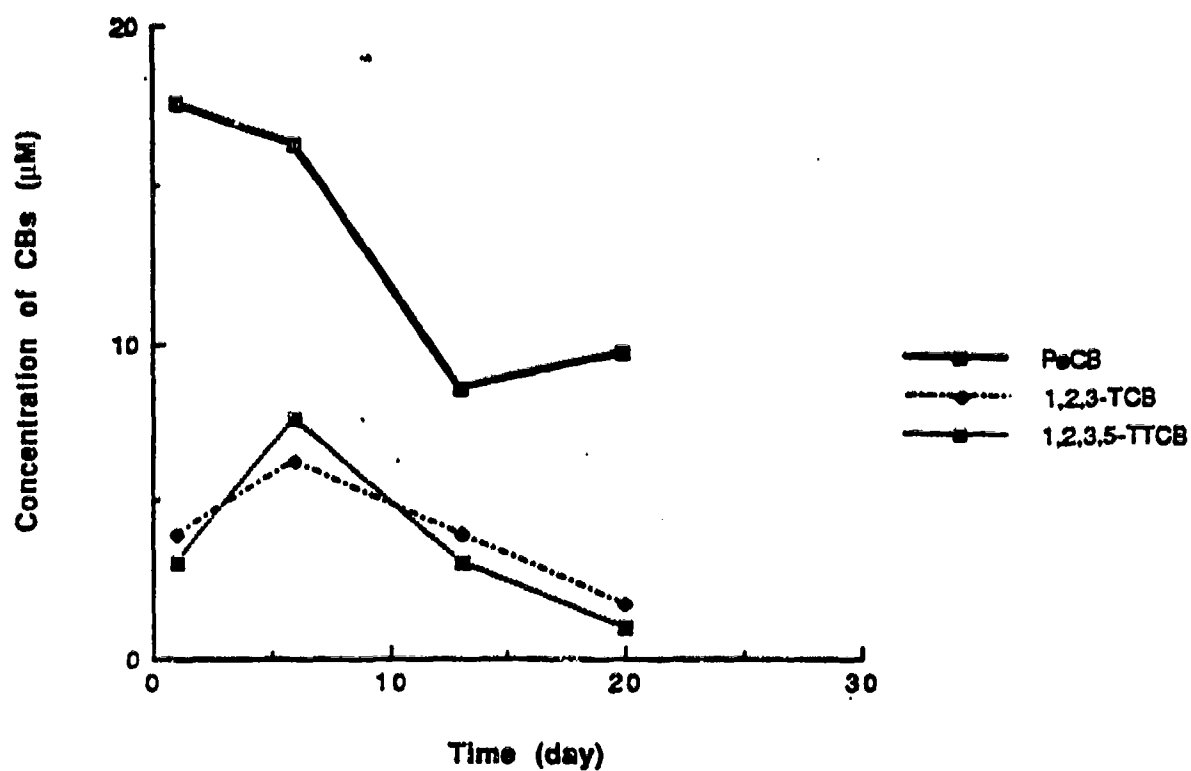


Figure 41. Reductive dechlorination of PeCB, 1,2,3,5-TTCB, and 1,2,3-TCB by the cells enriched on PeCB. The results from triplicate cultures were corrected for the background values measured in duplicate controls.

increase the rate of dechlorination either, although *p*-cresol is found as a contaminant in Pensacola site and is degraded by the indigenous microflora (Godsy et al., 1989). Since the aromatic substrates did not work, other electron donors -- acetate as a sole substrate, and a complex organic mixture containing acetate, yeast extract, and pepton -- were tested together with 1,2,3,5-TTCB or PeCB, dissolved in methanol. The cells from the enrichments were harvested and washed and inoculated into the medium with the various combinations of CBs and electron donors. The biotransformations of the CBs started after a 4-day lag. As shown in Table 11, the rate of the reductive dechlorination of 1,2,3,5-TTCB was higher in the cultures with complex organic substrates than in either the cultures with methanol alone, or the cultures with the addition of acetate. In general, the transformation was the slowest in the cultures containing acetate and methanol. A similar trend was observed with PeCB. Protein measurements in the cultures, however, indicated -- as expected -- that the growth was the fastest in the medium with complex organic additions (production of 9.5 mg L<sup>-1</sup> of protein during a 83-days incubation period), the slowest in the cultures that received only methanol (4.8 mg protein L<sup>-1</sup>), and in between these two values for the cultures in the medium with acetate plus methanol (5.5 mg L<sup>-1</sup>). These results indicate that acetate changes the composition of the microbial community so that the number of non-dehalogenating microorganisms increases relatively to the dehalogenating microorganisms. Other organic donors tested (yeast extract, pepton) support the dehalogenating activity.

To further investigate these phenomena, the cells were harvested and washed again and inoculated into the medium with the same combinations of substrates; this time, however, methanol was excluded, and the CBs were added as pure substances. As shown in Figure 42, the production of 1,3,5-TCB from 1,2,3,5-TTCB was the fastest in the complex medium, the next fastest in the medium without organic donor additions, and the slowest in the medium with the addition of acetate. These results support the previous observation that acetate indeed seems to inhibit dehalogenation by changing the composition of the microbial community, whereas yeast extract and pepton support dehalogenation. A similar trend was observed for the production of 1,3-DCB from PeCB, and 1,3,5-TCB from PeCB (Figure 43). Interestingly, however, the production of 1,2,3,5-TTCB from PeCB (Figure 44) showed an entirely different pattern, with acetate-amended cultures dechlorinating PeCB to 1,2,3,5-TTCB the fastest, and the complex organics-amended cultures the slowest and no different from the sterile controls. As observed before for PeCB, the microorganisms performing the initial dehalogenation of



Table 11. Rates of reductive dechlorination of 1,2,3,5-TTCB and production of 1,3,5-TCB by washed cells from the enrichments, with various electron donors<sup>a</sup>

Additions	Rates ( $\mu\text{M day}^{-1}$ )	
	Incubation Period (Days)	
	0-4	14-18
1,2,3,5-TTCB only <sup>b</sup>	$-(0.39 \pm 0.25)^c$	$-(2.27 \pm 0.50)$
1,2,3,5-TTCB + acetate <sup>d</sup>	$-(0.31 \pm 0.15)$	$-(1.56 \pm 0.31)$
1,2,3,5-TTCB + acetate + YE + pepton <sup>e</sup>	$-(0.21 \pm 0.20)$	$-(3.44 \pm 1.89)$
Autoclaved control (1,2,3,5-TTCB only)	$-(0.32 \pm 0.55)$	$-(0.19 \pm 4.92)$
1,3,5-TCB from		
1,2,3,5-TTCB only	$+(0.15 \pm 0.03)^f$	$+(7.92 \pm 1.44)$
1,2,3,5-TTCB + acetate	$+(0.10 \pm 0.12)$	$+(6.49 \pm 1.63)$
1,2,3,5-TTCB + acetate + YE + pepton	$+(0.04 \pm 0.07)$	$+(8.21 \pm 2.90)$
Autoclaved control (1,2,3,5-TTCB only)	$-(0.04 \pm 0.14)$	$+(0.01 \pm 0.24)$

<sup>a</sup> All the results are expressed as mean  $\pm$  standard deviation of triplicate active cultures or duplicate autoclaved controls. Traces of MCB and 1,3-DCB were also produced in active cultures during transformation (data not shown).

<sup>b</sup> All the cultures, including "1,2,3,5-TTCB only", contained methanol (used to dissolve the CB) as a potential electron donor.

<sup>c</sup> A negative sign means the compound concentration is declining (removal due to sorption and/or degradation).

<sup>d</sup> 0.33 mM sodium acetate was used.

<sup>e</sup> 2.5 mM sodium acetate, 1 g L<sup>-1</sup> of yeast extract (YE), and 0.1 g L<sup>-1</sup> of Bacto-pepton was used.

<sup>f</sup> A positive sign indicates that the compound concentration is increasing (due to desorption and/or production from the parent substrate).

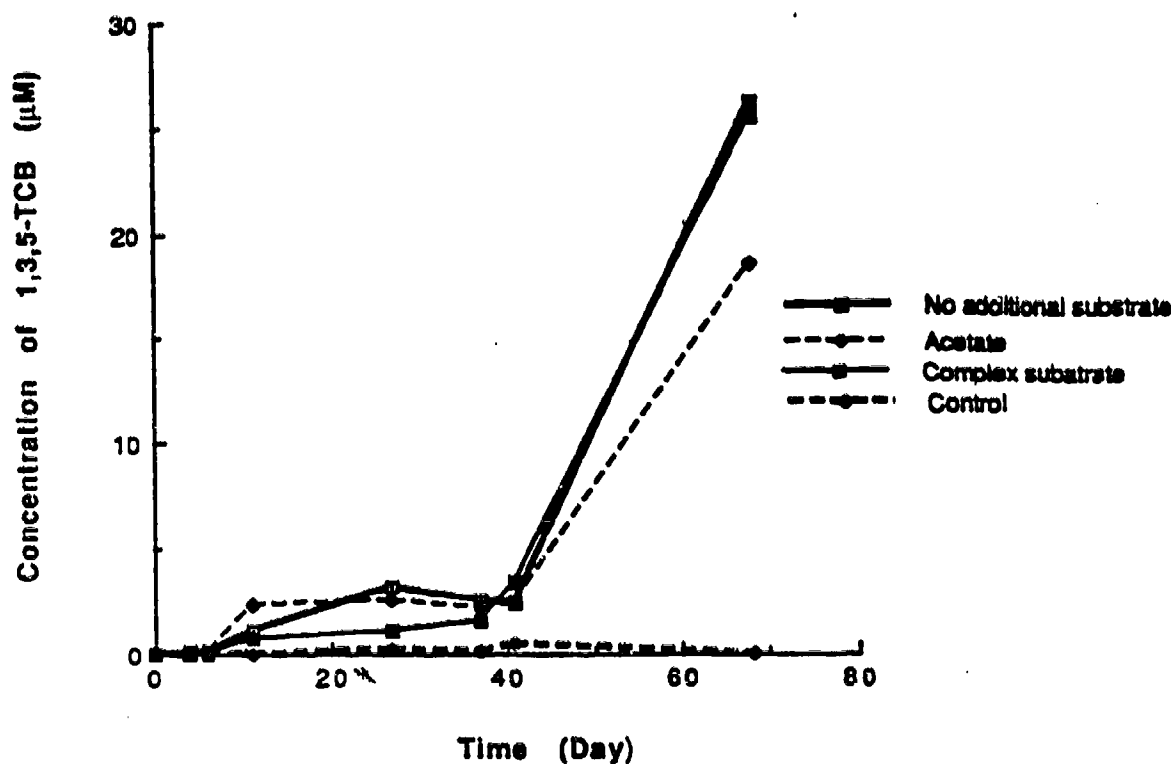


Figure 42. 1,3,5-TCB production from 1,2,3,5-TTCB in anaerobic enrichments with various electron donor additions (no additional substrate, or 0.33 mM sodium acetate, or 2.5 mM sodium acetate + 1 g L<sup>-1</sup> yeast extract + 0.1 g L<sup>-1</sup> Bacto-pepton). The results from triplicate cultures were corrected for the background values measured in duplicate controls

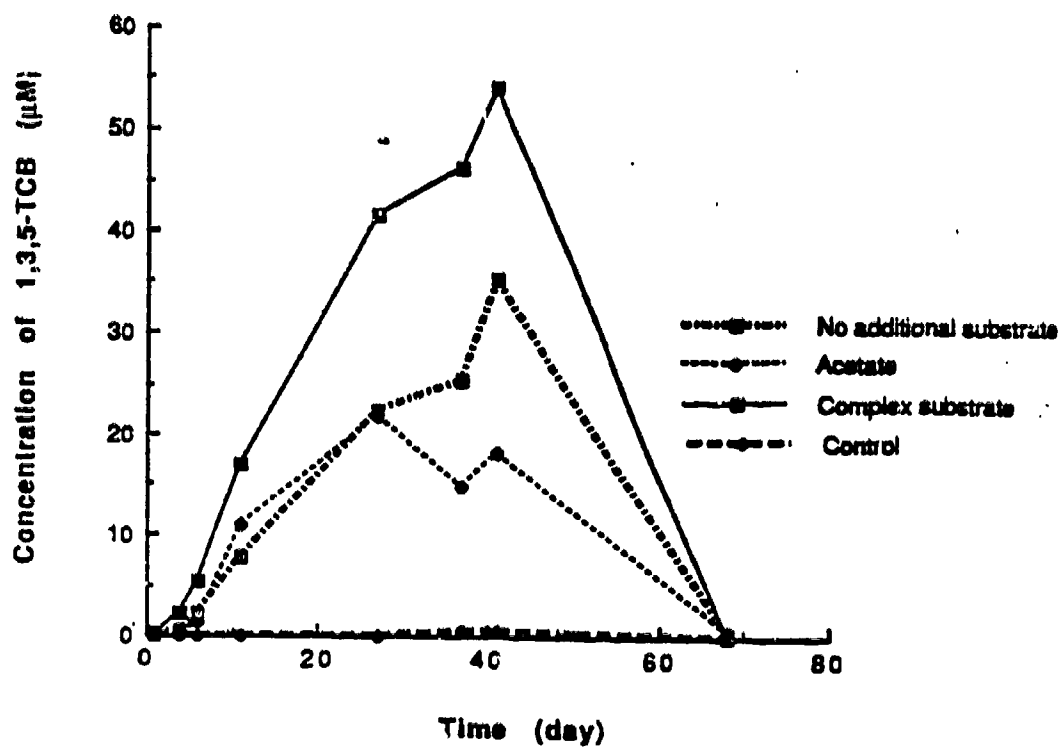


Figure 43. 1,3,5-TCB production from PeCB in anaerobic enrichments with various electron donor additions (no additional substrate, or 0.33 mM sodium acetate, or 2.5 mM sodium acetate + 1 g L<sup>-1</sup> yeast extract + 0.1 g L<sup>-1</sup> Bacto-pepton). The results from triplicate cultures were corrected for the background values measured in duplicate controls

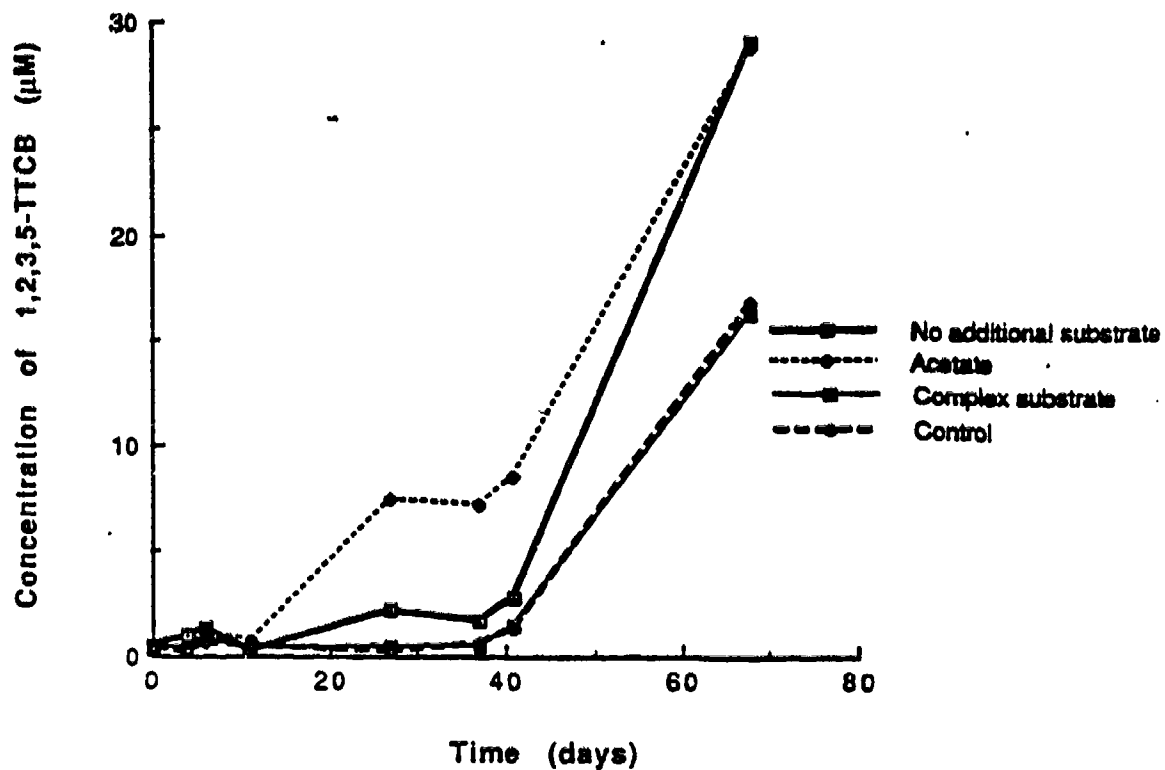


Figure 44. 1,2,3,5-TTCB production from PeCB in anaerobic enrichments with various electron donor additions (no additional substrate, or 0.33 mM sodium acetate, or 2.5 mM sodium acetate + 1 g L<sup>-1</sup> yeast extract + 0.1 g L<sup>-1</sup> Bacto-pepton). The results from triplicate cultures were corrected for the background values measured in duplicate controls

this compound seem to be entirely different from the organisms dehalogenating 1,2,3,5-TTCB or 1,3,5-TCB.

In summary, the types of organic compounds present in the subsurface can greatly influence the rates and pattern of dechlorination of CBs. Whereas aromatic compounds seem to have no influence, at least during the incubation periods on the order of 100 days, other organics can either slow down, or accelerate the dehalogenation process by acting as electron donors. It is interesting to note that all of the cells used in these experiments were packed with internal storage granules (poly-beta-hydroxybutyrate), which were obviously serving as electron sources in the dehalogenating process when no organic donors were added to the medium. Internal storage compounds are likely to be of a significant importance in the subsurface, by virtue of supporting electron-consuming microbiological processes in pollutant transformation.

## MIXTURES OF CHLORINATED ALIPHATIC SOLVENTS AND AROMATICS

### A. Traverse City Microcosms

The microcosms were set up with 75 g of preincubated aquifer material (24 days of preincubation in an anaerobic chamber), and 100 mL of prereduced defined mineral medium without vitamins. In one set of the experiments, 0.15 to 1.5 mg L<sup>-1</sup> of CT was added to the microcosms alone or in combination with 10 mg L<sup>-1</sup> of toluene, 10 mg L<sup>-1</sup> of ethylbenzene, or 200 mg L<sup>-1</sup> of benzoate. In the other set of the experiments, up to 10 mg L<sup>-1</sup> of PCE, alone or in combination with aromatic compounds, was used instead of CT. No other organic substrates were added to the microcosms. The incubation temperature was 35°C.

Figure 45A shows PCE transformation in the microcosms with the addition of toluene, and those that received no aromatic additions. There is no significant difference between those two sets of microcosms, suggesting that the presence of toluene did not influence PCE transformation. The findings of the experiments with the microcosms amended with various aromatic compounds are summarized in Table 12. Neither toluene, nor ethylbenzene accelerated PCE transformation; benzoate seemed to slow it down slightly. None of the aromatic hydrocarbons were degraded in the microcosms during the experimental period (data not shown). Benzoate was degraded slowly (3.53% in 50 days). Generally, the transformation of PCE was fast in all the microcosms. The rate of PCE transformation in e.g. toluene-amended microcosms was  $3.36 \pm 1.69$   $\mu\text{M PCE day}^{-1}$  during the first 7 days of incubation, increased to  $8.16 \pm 4.88$   $\mu\text{M day}^{-1}$  between the 8th and 9th day, and decreased to the initial levels late in incubation ( $3.24 \pm 0.40$   $\mu\text{M PCE day}^{-1}$  between days 222 and 236). TCE and 1,2-DCE were detected as the products of PCE transformation in all the active microcosms (Fig. 45B); traces of VC were also detected, but not quantified. The GC procedure that we used allowed for differentiation between isomers of DCE, because their retention times were different; the DCE found as a product from PCE in Traverse City microcosms, as well as in Pensacola microcosms, was the *trans*-1,2-DCE isomer. As evident from Fig. 45B, PCE was almost stoichiometrically converted to *trans*-1,2-DCE (within the constraints of experimental error). On the contrary, the autoclaved controls did not display a significant removal of PCE (Fig. 45A); low concentrations of TCE were found in the controls, but neither DCE nor VC were

Figure 45. PCE transformation in Traverse City microcosms. A: PCE transformation in the presence of toluene ("PCE + Toluene"), and in its absence ("PCE only"). Each data point in the active microcosm curves is the mean of measurements in triplicate microcosms. Each data point in the "Control" curve is the mean of measurements in duplicate autoclaved controls. PCE was respiked to active microcosms on day 8, to both active microcosms and controls on day 81, and to active microcosms and one of the two controls on day 222. B: PCE transformation and production of mean of triplicate measurements in active microcosms. Controls are not shown; they produced low concentrations of TCE, but no DCE.

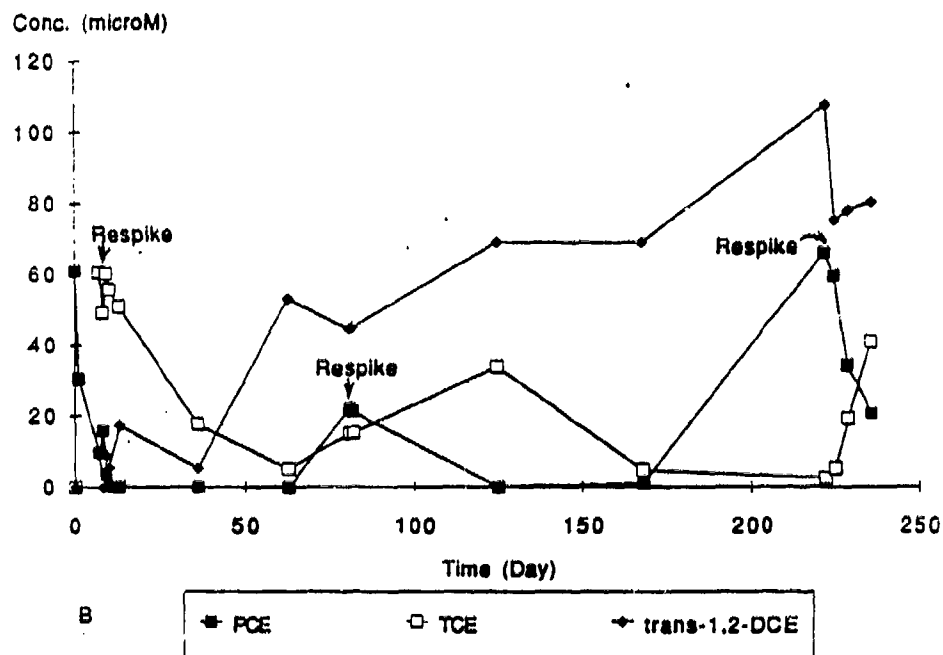
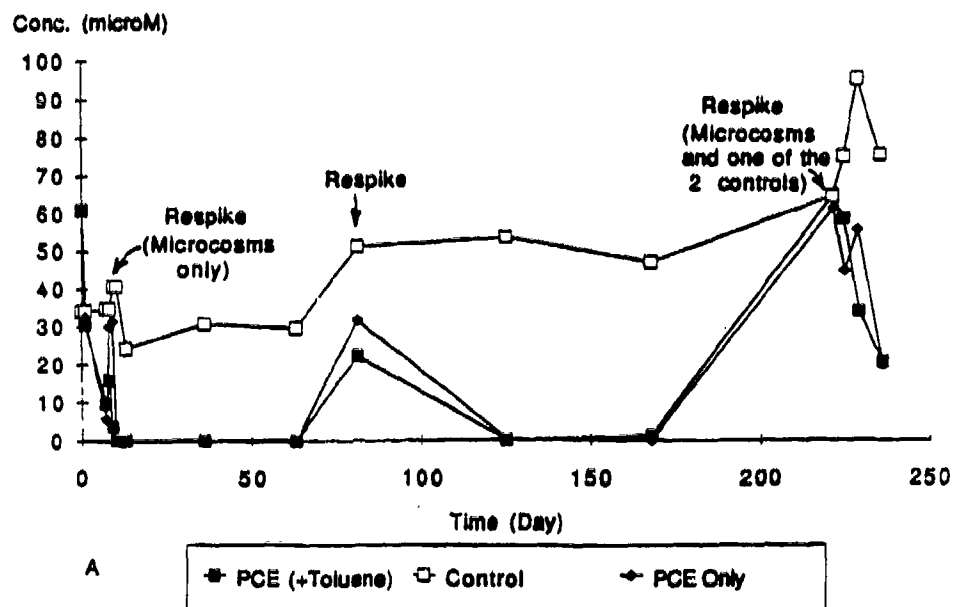




Table 12. Rates of PCE transformation ( $\mu\text{M day}^{-1}$ )<sup>a</sup> in Traverse City microcosms in the presence or absence of various aromatic compounds, during the first seven days of incubation

Aromatic compound added	Active microcosms	Autoclaved controls
None	$-(4.43 \pm 0.95)^b$	Not Available
Toluene (10 mg L <sup>-1</sup> )	$-(3.36 \pm 1.69)$	$+(0.091 \pm 0.31)^c$
Ethylbenzene (10 mg L <sup>-1</sup> )	$-(5.01 \pm 0.87)$	$+(0.07 \pm 1.77)$
Benzoate (200 mg L <sup>-1</sup> )	$-(2.00 \pm 1.02)$	$+(0.187 \pm 0.92)$

<sup>a</sup> The rates are given as mean  $\pm$  standard error (95% confidence interval), and are the results of measurements in triplicate active microcosms, and duplicate autoclaved controls.

<sup>b</sup> A negative sign means that the compound concentration in the microcosm headspace is declining (removal due to sorption and/or degradation).

<sup>c</sup> A positive sign means that the compound concentration in the microcosm headspace is increasing (due to desorption).

formed. Some sorption of PCE, presumably to aquifer solids, occurred immediately upon spiking of PCE to the controls (Fig. 45A).

Transformation of CT by Traverse City microcosms was similar to the PCE transformation in that there was no significant difference in either the rate or extent of transformation between the microcosms amended with aromatic hydrocarbons, and those that received no amendments; furthermore, benzoate again seemed to slow down the transformation. However, the rates of CT biotransformation were approximately two orders of magnitude lower than the rates of PCE biotransformation, and abiotic transformation in the controls was more pronounced than in the case of PCE. CF was the only CT transformation product detected in both active microcosms and controls, but the rate of CF formation was higher in the active microcosms (Fig. 46). CF produced could not account for all of the CT removed.

None of the aromatic hydrocarbons were degraded during the incubation period; benzoate was transformed very slowly (0.5% in 50 days). However, toluene alone (in the absence of either CT, or PCE) was degraded slowly by Traverse City microflora ( $10 \text{ mg L}^{-1}$  [100%] in 156 days of incubation).

#### **B. Pensacola Microcosms**

The active microcosms and autoclaved controls consisted of 50 g of preincubated aquifer material (20 days of preincubation in an anaerobic chamber) and 100 mL of prereduced defined medium. They were used for studies of PCE ( $10 \text{ mg L}^{-1}$ ) and CT ( $1.5 \text{ mg L}^{-1}$ ) transformation, alone or in the presence of  $10 \text{ mg L}^{-1}$  toluene. No other organic compounds were added to the microcosms. The incubation temperature was  $35^\circ\text{C}$ . Table 13 shows transformation rates for CT and PCE during certain incubation periods. Although there were no initial acclimation lags, higher transformation rates for both CT and PCE later in incubation (Table 13) indicate either acclimation of the microflora, or development of denser biomass. Similar to the results from Traverse City microcosms, the PCE transformation rates were higher (about an order of magnitude) than the CT transformation rates, and abiotic transformation of CT was more pronounced than that of PCE. Furthermore, toluene was not degraded during the incubation period, not even in the microcosms without any chlorinated organics (data not shown), and it did not enhance the transformation of either PCE, or

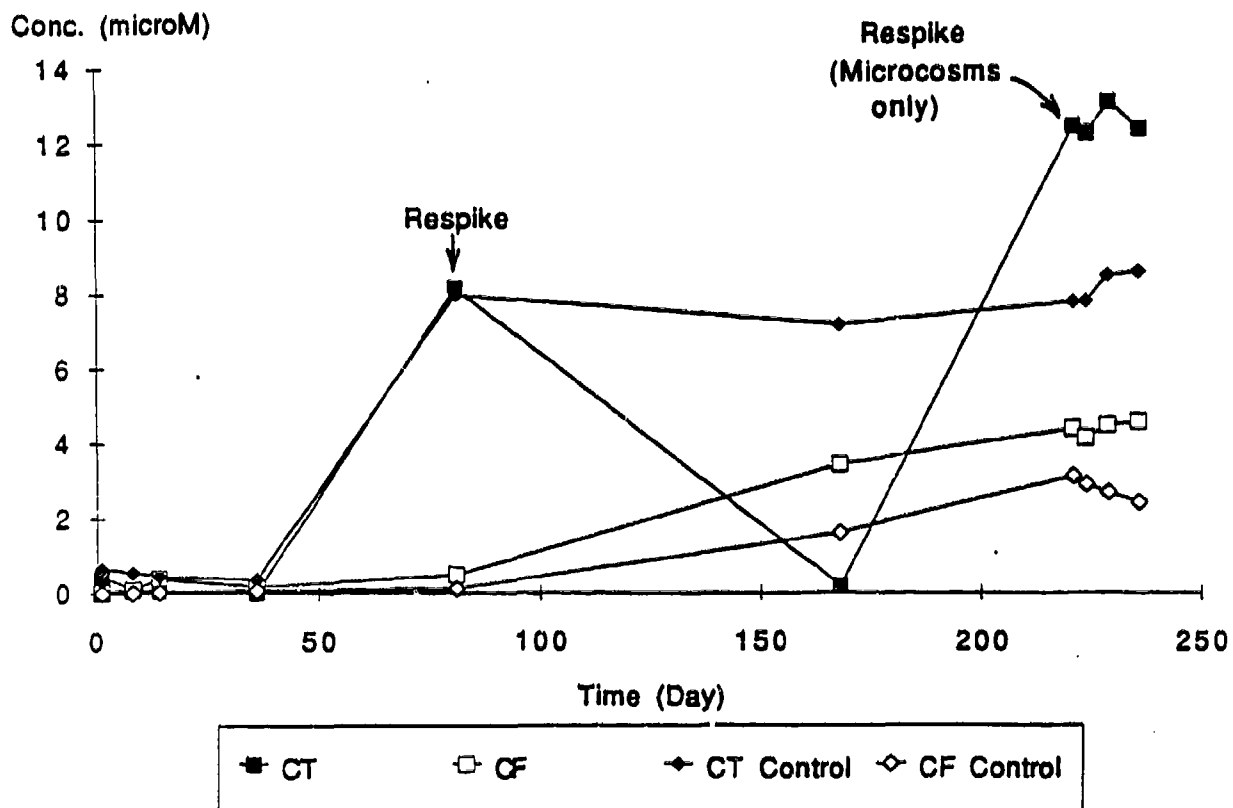


Figure 46. CT transformation in Traverse City microcosms amended with toluene.

Each data point in the active microcosm curves for CT transformation and CF production is the mean of measurements in triplicate microcosms. Each data point in the "Control" CT and CF curves is the mean of measurements in duplicate autoclaved controls. CT was respiked to both active microcosms and autoclaved controls on day 81, and to active microcosms only on day 221.

Table 13. Rates of CT and PCE transformation ( $\mu\text{M day}^{-1}$ )<sup>a</sup> in Pensacola microcosms, in the presence or absence of toluene

Chlorinated solvents and aromatic compounds added	Active microcosms/ Incubation period (days)	Autoclaved controls/ Incubation period
PCE alone	$-(1.45 \pm 0.34)^b/1-40$ days $-(2.16 \pm 0.34)/116-131$ d	Not Available
PCE + Toluene	$-(1.14 \pm 0.38)/1-38$ d $-(1.32 \pm 0.42)/116-139$ d	$+(0.31 \pm 0.49)^c/1-38$ d $+(1.49 \pm 0.41)/115-139$ d
CT alone	$-(0.18 \pm 0.022)/1-26$ d $-(0.33 \pm 0.024)/$ 107-122 d	Not available
CT + Toluene	$-(0.17 \pm 0.011)/27-38$ d $-(0.202 \pm 0.06)/$ 107-130 d	$-(0.09 \pm 0.05)/27-38$ d $-(0.012 \pm 0.117)/$ 107-130 d

<sup>a</sup> The rates are given as mean  $\pm$  standard error (95% confidence interval), and are the results of measurements in triplicate active microcosms, and duplicate autoclaved controls.

<sup>b</sup> A negative sign means that the compound concentration in the microcosm headspace is declining (removal due to sorption and/or degradation).

<sup>c</sup> A positive sign means that the compound concentration in the microcosm headspace is increasing (due to desorption).

CT. The only transformation product from CT that was detected was CF (Fig. 47). The mass of CF produced did not account for all of the CT removed. PCE was transformed to TCE and *trans*-1,2-DCE (Fig. 48). Trace amounts of VC were also found in the PCE-amended microcosms. It should be noted that TCE and DCE were detected only after 120 days of incubation, although PCE removal occurred throughout the incubation period (Fig. 48). Only traces of TCE, and no DCE or VC were detected in the autoclaved controls amended with PCE, after 115 days of incubation. However, controls did exhibit instantaneous sorption of PCE upon addition, and subsequent slow desorption; the aqueous concentration of PCE reached its maximum after 130 days of incubation (data not shown).

Pensacola microcosms actively produced gas, including methane. Table 14 shows CH<sub>4</sub> concentration (uM) in the headspace after 151 days of incubation. Autoclaved controls are not presented, because they formed negligible amounts of methane. It is interesting to note that the microcosms amended with CT alone produced very little CH<sub>4</sub>, whereas the microcosms amended with PCE alone and those with PCE + toluene formed about the same amount of CH<sub>4</sub>; this amount was greater than in the microcosms amended with toluene alone.

### C. Tyndall Airforce Base Microcosms.

The microcosms consisted of 15 g of preincubated and washed aquifer material and 120 mL of prereduced defined medium. They were incubated at 35°C (microcosms with CT), or room temperature (microcosms with PCE). The transformation of CT (0.5-2.0 mg L<sup>-1</sup>) was studied in the presence of 10 to 20 mg L<sup>-1</sup> toluene or 100 mg L<sup>-1</sup> benzoate, or in their absence. In addition to these organic substrates, a low concentration of acetate (CP medium, as specified in the Materials and Methods) was amended to one group of the microcosms, a high concentration of acetate plus yeast extract and Bacto-peptone (CR medium) were added to a parallel group of microcosms, and no accessory organic amendments were added to the third group of microcosms. Table 15 shows the rates of transformation of CT with various combinations of organic additions. The presence of 0.33 mM sodium acetate increased the rate of CT transformation slightly. With 25 mM acetate and in the presence of accessory organic substrates (CR medium), the rate of CT removal was increased even further (Fig. 49). Toluene had no influence on CT transformation

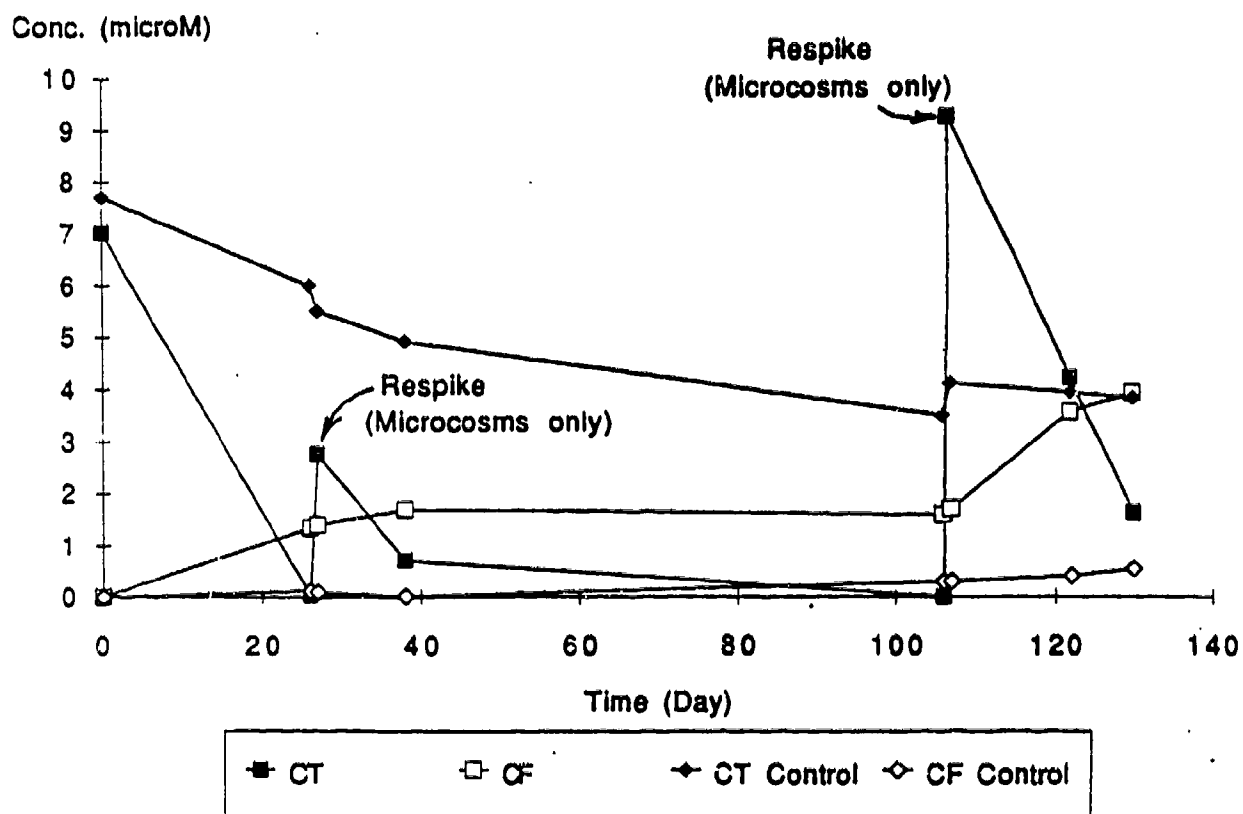


Figure 47. CT transformation in Pensacola microcosms unamended with any aromatics. Each data point in the active microcosm curves for CT transformation and CF production is the mean of measurements in triplicate microcosms. Each data point in the "Control" CT and CF curves is the mean of measurements in duplicate autoclaved controls. CT was respiked only to the active microcosms on days 27 and 107.

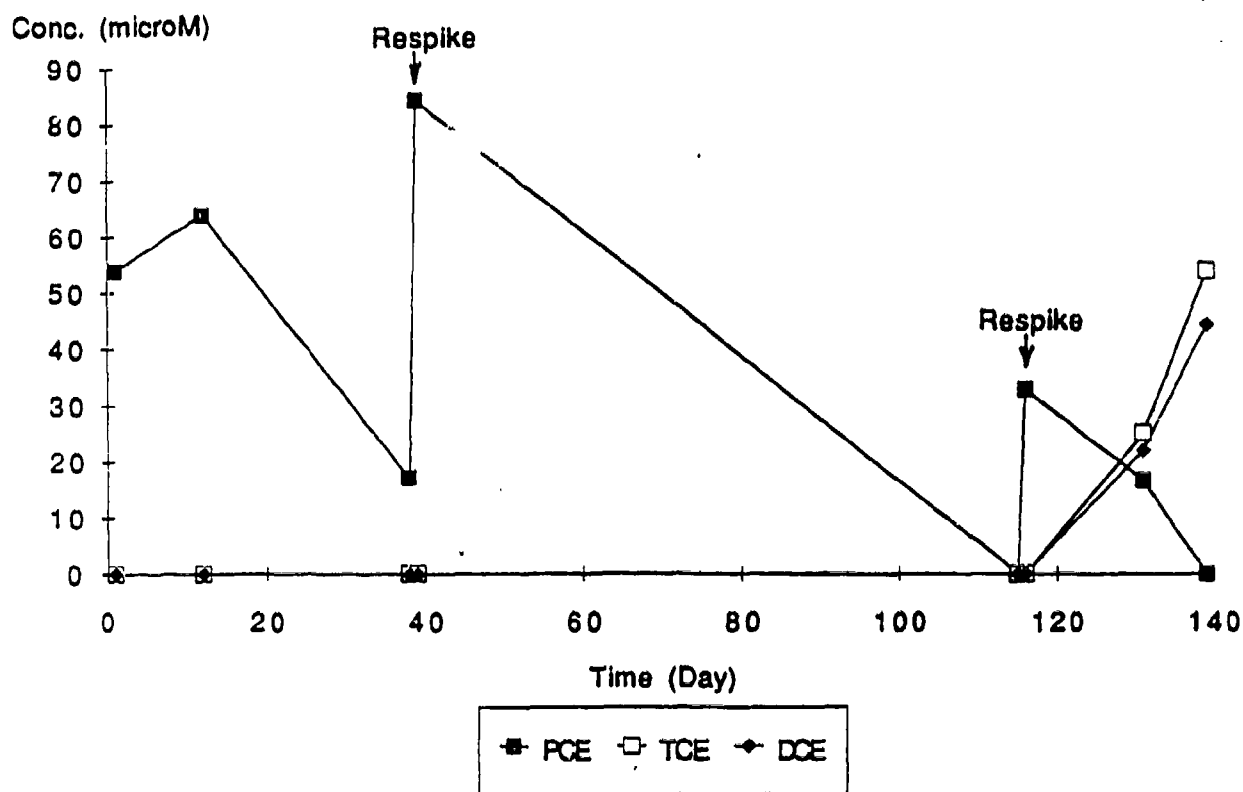


Figure 48. PCE transformation and production of TCE and *trans*-1,2-DCE in Pensacola microcosms amended with 10 mg L<sup>-1</sup> toluene. Each data point is the mean of measurements in triplicate active microcosms. Controls are not shown; the transformation of PCE in the controls was insignificant. The active microcosms were respiked on days 39 and 116.

Table 14. Methane concentration ( $\mu\text{M}$ )<sup>a</sup> in the headspace of Pensacola microcosms after 151 days of incubation.

Compounds added	Active microcosms
Toluene alone	308.1
CT alone	2.6
CT + Toluene	106.5
PCE alone	470.7
PCE+Toluene	477.9

<sup>a</sup> The concentrations are given as mean of measurements in triplicate active microcosms.



Table 15. Rates of CT transformation in Tyndall Airforce Base microcosms in the presence or absence of various accessory organics

Conditions and organic compounds added	Transformation rate ( $\mu\text{M day}^{-1}$ ) <sup>a</sup>	Time period (days of incubation)
CT only	$-(0.39 \pm 0.031)^b$	0-4
	$-(0.35 \pm 0.098)$	6-8
CT + CP <sup>c</sup>	$-(0.42 \pm 0.075)$	0-13
CT + Benzoate	$-(0.29 \pm 0.17)$	0-4
	$-(0.295 \pm 0.083)$	6-8
CT + CP + Benzoate	$-(0.45 \pm 0.029)$	0-4
	$-(0.405 \pm 0.030)$	6-8
CT + CR <sup>d</sup> + Benzoate	$-(0.48 \pm 0.05)$	0-4
	$-(0.71 \pm 0.43)$	6-8
CT + CP + Toluene	$-(0.44 \pm 0.069)$	0-13
CT + CR + Toluene	$-(0.63 \pm 0.15)$	0-13
CT only, Autoclaved Control	$-(0.18 \pm 0.038)$	0-13
CT + CR + Benzoate, Autoclaved Control	$-(0.047 \pm 0.030)$	0-4
	$-(0.023 \pm 0.10)$	6-8
CT + CR + Toluene, Autoclaved Control	$-(0.25 \pm 0.030)$	0-13

Table 15. -- continued

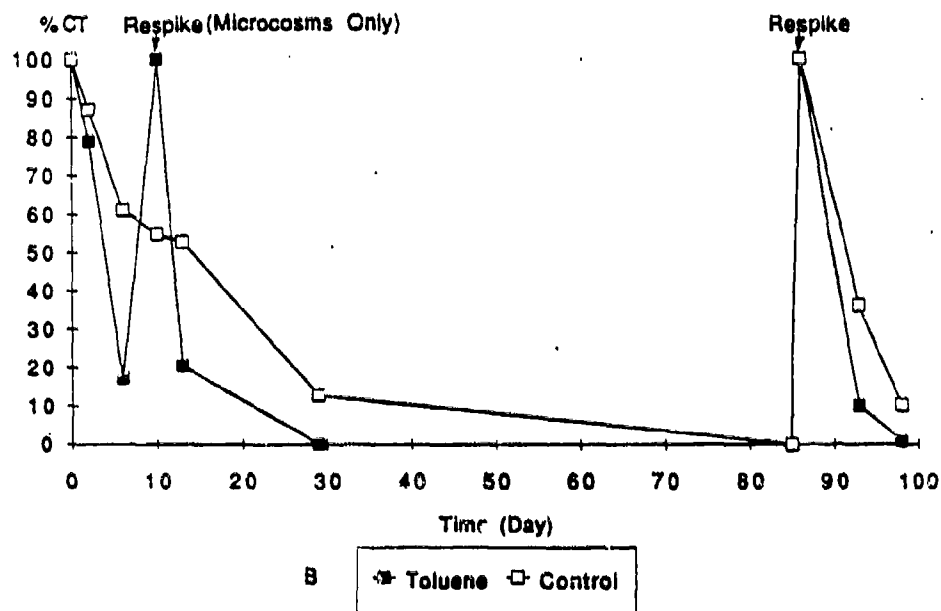
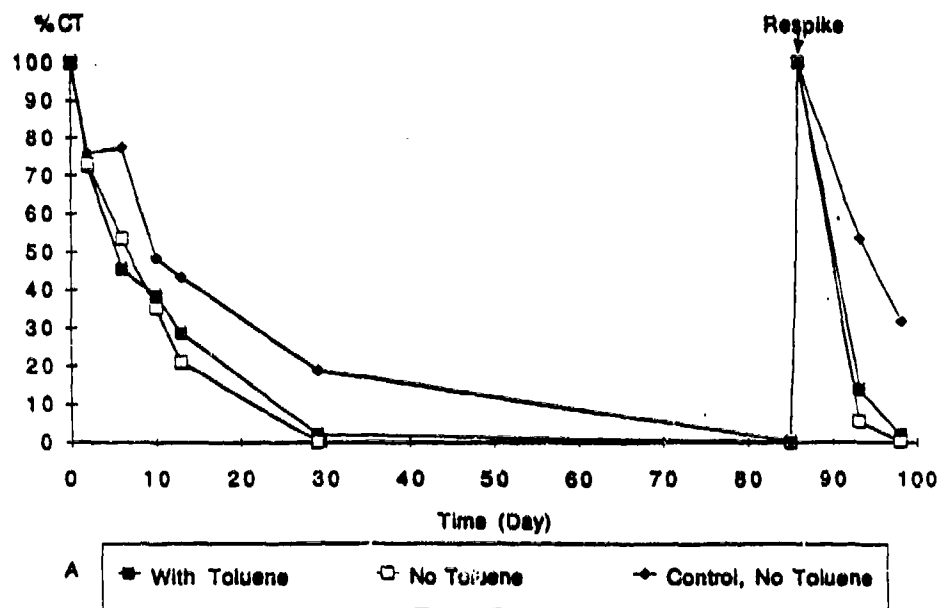
<sup>a</sup> The rates are given as mean  $\pm$  standard error (95% confidence interval), and are the results of measurements in triplicate active microcosms, and duplicate autoclaved controls.

<sup>b</sup> A negative sign means that the compound concentration in the microcosm headspace is declining (removal due to sorption and/or degradation).

<sup>c</sup> CP = addition of 0.33 mM sodium acetate.

<sup>d</sup> CR = addition of 25 mM sodium acetate, 1 g L<sup>-1</sup> of yeast extract, and of 0.1 g L<sup>-1</sup> Bacto-peptone.

Figure 49. CT transformation in Tyndall Airforce Base (TAF) microcosms. Each data point in the active microcosm curves is the mean of measurements in triplicate microcosms. Each data point in the "Control" curves is the mean of measurements in duplicate autoclaved controls. A: Comparison of CT transformation in the presence of toluene ("With Toluene") and in the absence thereof ("No Toluene"), in CP medium containing 0.33 mM acetate. B: CT transformation in the presence of toluene, in CR medium containing 25 mM acetate, 1 g L<sup>-1</sup> of yeast extract, and 0.1 g L<sup>-1</sup> of Bacto-peptone.



(Table 15, Fig. 49). Benzoate seemed to have reduced the rate of CT transformation slightly (Table 15), but degradation of benzoate was not observed during the experimental period. Toluene was not found to biodegrade either. CF was detected as a product from CT, but it was not quantitated. Autoclaved controls also exhibited CT removal, but the rates were lower than in the active microcosms (Table 15, Fig. 49).

The transformation of PCE (10 mg L<sup>-1</sup>) in TAF microcosms was evaluated in the presence of toluene, benzoate, or phenol (100 mg L<sup>-1</sup>). No other organic compounds were added. PCE was not transformed at all in the presence of either benzoate or phenol, but was transformed slowly ( $0.013 \pm 0.066$   $\mu\text{M day}^{-1}$ , measured in 85 days) in the presence of toluene, and so was the toluene itself ( $0.37 \pm 0.16$   $\mu\text{M day}^{-1}$ , measured in 85 days). Fig. 50 presents the relationship between PCE transformation and toluene degradation. Contrary to all the other experiments described above, the transformation of PCE could possibly be related to that of toluene, and there seemed to be a dependence between these two processes. Neither PCE, nor toluene were transformed at all in the autoclaved controls. The products of PCE transformation were not studied in this experiment.

#### D. Stable Methanogenic Consortia Degrading Toluene.

CT (1 mg L<sup>-1</sup>) or PCE (10 mg L<sup>-1</sup>) were added along with 15 mg L<sup>-1</sup> toluene to stable suspended consortia derived from the Pensacola microcosms. No other organic compounds were present. Fig. 51 shows CT transformation and toluene degradation in the consortia. Although CT was completely removed in 80 days, toluene stagnated until about 160 days, and was then slowly degraded (two months needed for complete removal). Low concentrations of CF, a transformation product from CT, were detected (0.26  $\mu\text{M}$  CF on day 80, from the initial CT concentration of 5  $\mu\text{M}$ ). CF persisted also later in the incubation period (data not shown). Autoclaved controls transformed CT at rates one order of magnitude lower than the active consortia, and only 0.04  $\mu\text{M}$  of CF was formed from 5  $\mu\text{M}$  of CT on day 80. On the other hand, PCE was not transformed at all, either biotically or abiotically, and its presence did not influence toluene degradation. Table 16 shows the conversion of [<sup>14</sup>C]ring-toluene to <sup>14</sup>CO<sub>2</sub> during a month of incubation, in the presence or absence of chlorinated aliphatics: the amount of <sup>14</sup>CO<sub>2</sub> produced was the same in the

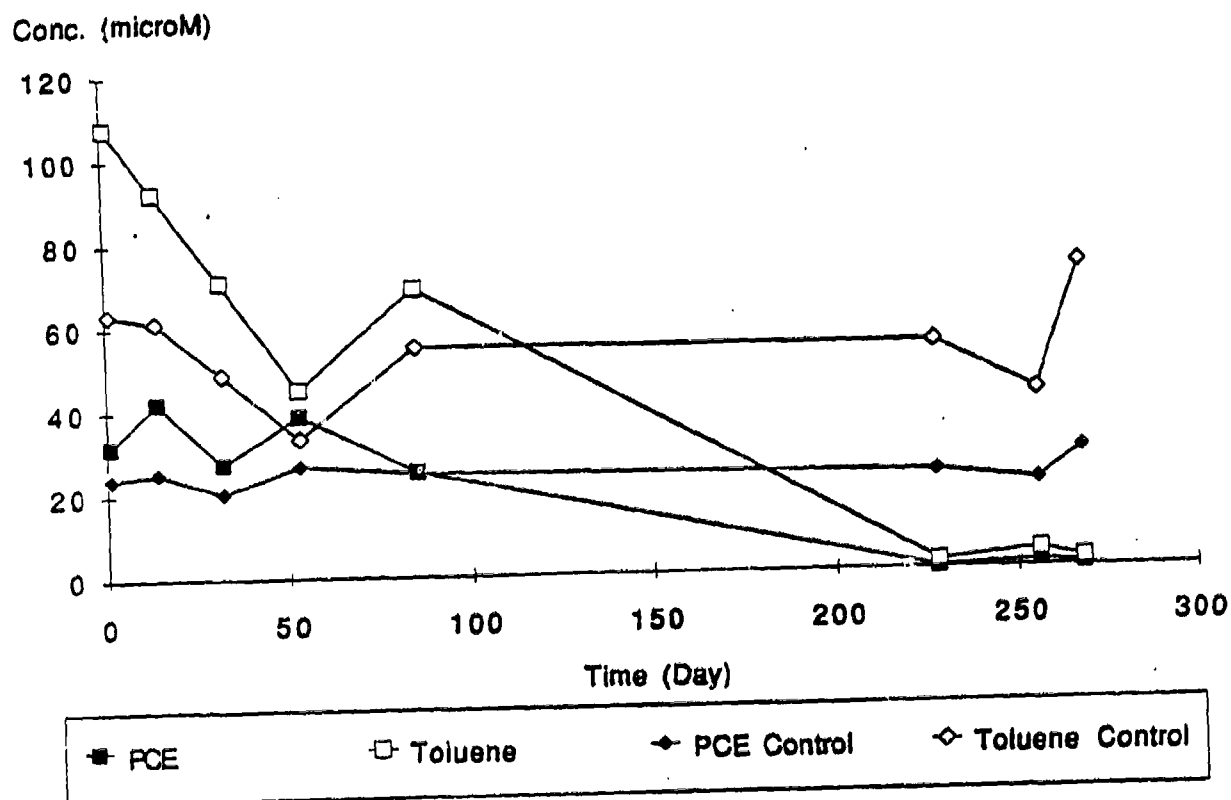


Figure 50. PCE transformation in the presence of toluene in TAF microcosms. Each data point in the active microcosm curves is the mean of measurements in triplicate microcosms. Each data point in the "Control" curves is the mean of measurements in duplicate autoclaved controls.

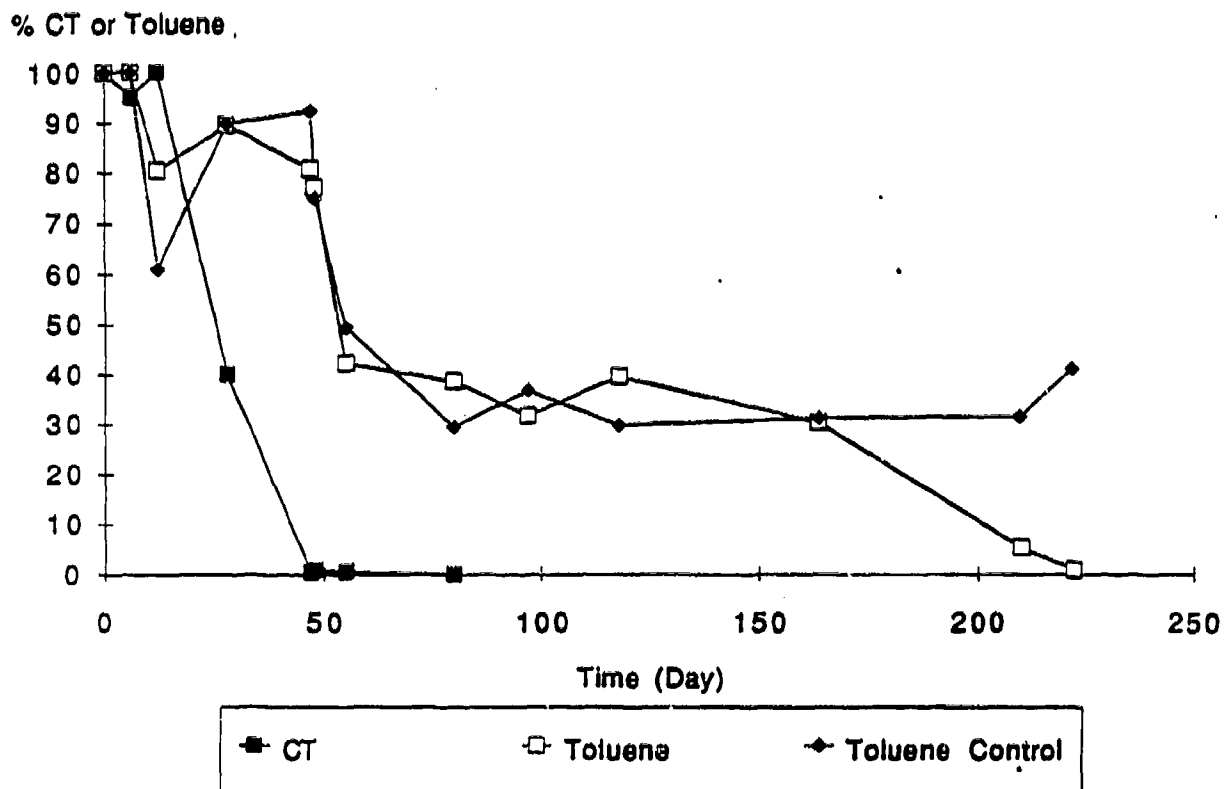


Figure 51. CT transformation and toluene degradation by stable methanogenic toluene-degrading consortia. "Toluene Control" = toluene behavior in autoclaved controls containing CT and toluene. The CT control is not shown; CT transformation rate was an order of magnitude lower in the autoclaved controls than in the active cultures. Each data point in the active culture curves is the mean of measurements in triplicate cultures. Each data point in the "Toluene Control" curve is the mean of measurements in duplicate autoclaved controls.

Table 16. Conversion of [ $^{14}\text{C}$ ]ring-toluene to  $^{14}\text{CO}_2$  in stable methanogenic consortia degrading toluene after 36 days of incubation (static, 35°C)

Substrates	% [ $^{14}\text{C}$ ]Toluene converted to $^{14}\text{CO}_2^a$
Toluene alone	78.1%
Toluene + PCE	78.55%
Toluene + CT	0%

<sup>a</sup> The results shown are the mean of measurements in triplicate active consortia. Autoclaved controls formed no  $^{14}\text{CO}_2$  from [ $^{14}\text{C}$ ]toluene.



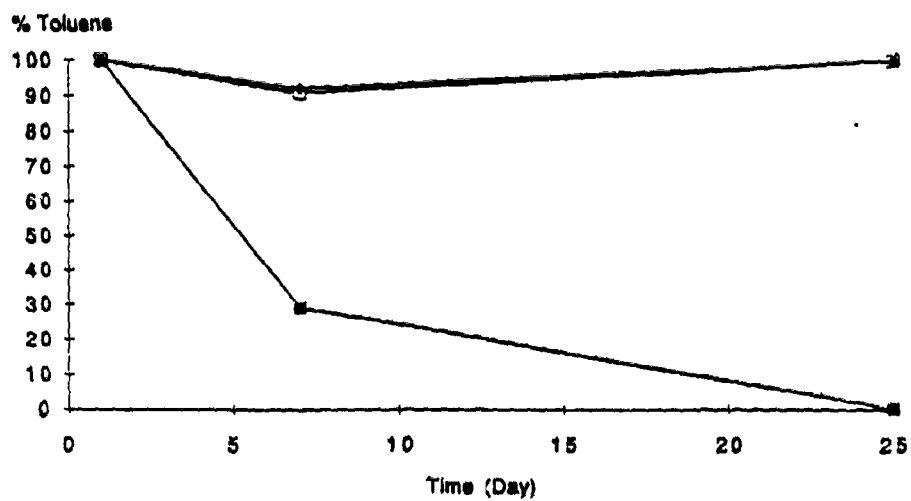
presence of PCE and in its absence, whereas CT completely inhibited toluene conversion to CO<sub>2</sub>.

In order to find out whether the product of CT transformation, CF, was inhibitory to toluene degradation, the toluene consortia were amended with 125  $\mu$ M toluene and 70  $\mu$ M CF. As shown in Fig. 52A, CF completely shut down the toluene degradation. On the contrary, 80  $\mu$ M TCE, 70  $\mu$ M *trans*-1,2-DCE, and 1.0  $\mu$ M VC (potential PCE transformation products under anaerobic conditions) were not significantly inhibitory to toluene degradation (Fig. 52B). Table 17 shows methane production data that support these findings: only CF exerted almost complete inhibition on CH<sub>4</sub> formation from toluene.

## E. Summary

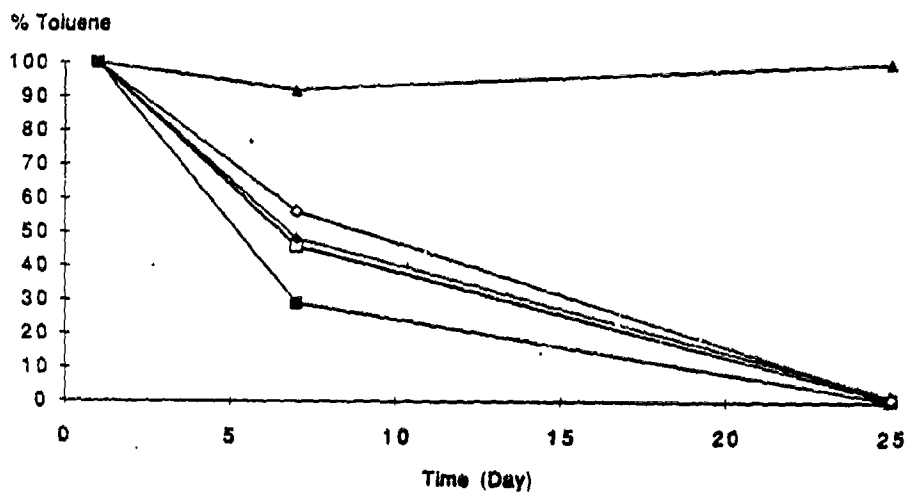
The transformation of CT occurred with all of the microbial inocula tested (as well as in the autoclaved controls), and CF was the only detected transformation product. Therefore, it can be stated that the initial transformation of CT was a reductive dechlorination. Since the CF produced could not account for all of the CT removed, it is likely that other products were also formed, that were not identified by the analytical techniques used. A range of additional products from CT had been reported by other authors previously (Bouwer and McCarty, 1983; Galli and McCarty, 1989). The transformation of CT in our microcosms occurred without a lag, but there was an acclimation lag (20 days) before the onset of transformation in the toluene-degrading methanogenic consortia that had no other external electron donors for CT reduction but H<sub>2</sub> from the anaerobic headspace (CT was transformed before the toluene degradation started, indicating that toluene was not the likely electron donor for CT transformation). The estimated half-life for CT in active microcosms was 8-10 days, whereas in stable methanogenic cultures it was about 25 days; these numbers are similar to the results of Wood et al. (1985) for anaerobic aquifer microcosms, and of Bouwer and McCarty (1989) for sludge-derived consortia. The probable electron donors for CT reduction in our experiments were the unidentified organic compounds present on the aquifer solids, or hydrogen present in the anaerobic atmosphere, or endogenous reserves in microbial cells (stable methanogenic consortia). In TAF microcosms, the addition of acetate or complex organics (yeast extract, peptone) increased the rate of reductive dechlorination of CT. Acetate (Bouwer and McCarty,

Figure 52. The influence of CT and PCE transformation products on toluene degradation by stable methanogenic consortia. A: The influence of CF. B: The influence of TCE, *trans*-1,2-DCE, and VC. Each data point in the active culture curves is the mean of measurements in triplicate cultures. Each data point in the "Control" curves is the mean of measurements in duplicate autoclaved controls.



A

■ Toluene Alone □ Toluene + CF ▲ Control



B

■ Toluene Alone □ Toluene + TCE ▲ Toluene + DCE  
◇ Toluene + VC ★ Control

Table 17. Methane concentration ( $\mu\text{M}$ ) in the headspace of stable toluene-degrading methanogenic consortia after 58 days of incubation, in the presence or absence of CT and PCE transformation products<sup>a</sup>

Organic compounds added	Active consortia	Autoclaved controls
Toluene alone	172.4	0.4
Toluene + 80 $\mu\text{M}$ TCE	214.7	0.4
Toluene + 70 $\mu\text{M}$ DCE	81.4	0.7
Toluene + 1 $\mu\text{M}$ VC	186.4	0.4
Toluene + 70 $\mu\text{M}$ CF	1.6	0.3

<sup>a</sup> The results shown are the mean of measurements in triplicate active consortia, and duplicate autoclaved controls.

1983; Vogel and McCarty, 1985), as well as complex organic substrates such as yeast extract and tryptone (Galli and McCarty, 1989), had been shown previously to be good electron donors for reductive dechlorination under anaerobic conditions.

No connection could be established between aromatic hydrocarbon degradation and CT transformation in our experiments. Two of the tested inocula (Pensacola and TAF) did not degrade toluene at all during the incubation period observed (140 days). The other two inocula did: Traverse City microcosms degraded toluene alone (but not in the presence of CT), whereas stable methanogenic toluene-degrading consortia amended with CT degraded toluene, but only after a lag that was apparently caused by CT transformation (Fig. 51, Table 16). CO<sub>2</sub> and CH<sub>4</sub> production were inhibited in CT-amended microcosms (Tables 14 and 16). Our studies indicate that it is possibly CF, rather than CT, that acts as an inhibitor of toluene degradation (Fig. 52A, Table 17). The inhibitory effect of CF on methanogenic systems had been demonstrated before (Swanwick and Foulkes, 1971; Yang and Speece, 1986), and it had been reported that the inhibition exerted by CF was much stronger than the inhibition by CT (Swanwick and Foulkes, 1971). Furthermore, it had been shown that CF inhibited terminal methane formation steps in methanogens (Gunsalus and Wolfe, 1978). In complex methanogenic communities, inhibition of methanogenesis could cause negative feedback on earlier members of the food chain and eventually stop the degradation of an organic substrate, such as toluene in our experiments. Yang and Speece (1986) had suggested that methanogenic activity could be recovered in mixed methanogenic cultures exposed to CF -- not because of the disappearance of CF with time, but because of acclimation of the cultures to CF. Some of our results suggest that the same phenomenon might be occurring here (e.g., Fig. 51 -- toluene degradation in methanogenic consortia).

The results of the experiments with PCE indicate that this compound was reductively dechlorinated to a sequence of products -- TCE, *trans*-1,2-DCE, and VC -- in all the aquifer microcosms tested. PCE was not transformed by the suspended toluene-degrading methanogenic consortia. There was no lag before the onset of transformation in Traverse City microcosms, but in the other two cases -- Pensacola and TAF microcosms -- acclimation lags were observed (15 and 50 days, respectively). This indicates differences in the composition of microbial communities in different ground water aquifers; in the case of TAF microcosms, a low initial biomass concentration could have contributed also to the duration of the lag. Only

traces of VC were formed in the active aquifer microcosms, suggesting that reductive dechlorination of DCE was slower than that of PCE or TCE. Suflita et al. (1988) also observed accumulation of DCE, and only traces of VC, in PCE-transforming methanogenic and sulfate-reducing aquifer microcosms. These data agree with the available literature on transformation mechanisms for halogenated aliphatics, which states that less chlorinated (less oxidized) aliphatic compounds are transformed more slowly than highly chlorinated ones under reducing conditions (Vogel and McCarty, 1985; Vogel et al., 1987). The DCE isomer produced in our microcosms was *trans*-1,2-DCE. This is similar to the results of Freedman and Gossett (1989), for PCE transformation in methanogenic cultures derived from digested sludge. However, our findings differ from the results of Parsons et al. (1984, 1985), Parsons and Lage (1985), and Barrio-Lage et al. (1986), who observed the predominance of *cis*-isomer of DCE from PCE transformation in anaerobic aquifer microcosms.

The rate of PCE transformation in the aquifer microcosms (except TAF microcosms) was one to two orders of magnitude higher than that of CT. This could be explained partially by the higher initial concentrations of PCE used (60  $\mu$ M, vs. 1-10  $\mu$ M CT), but also by the differences in the microflora participating in transformation of PCE, or CT. The estimated microbiological half-life for PCE in Traverse City and Pensacola microcosms was on the order of 1.5 to 10 days, which is in the ballpark with the published data on methanogenic transformation of PCE by sludge-derived microorganisms (Bouwer and McCarty, 1983; Vogel and McCarty, 1985). The half-life of PCE in TAF microcosms (approximately 100 days) was closer to the published data for anaerobic aquifer microcosms (Parsons and Lage, 1985; Wood et al., 1985). The electron donors for the reductive dechlorination of PCE in our microcosms were probably unidentified organic compounds on the aquifer solids, and/or hydrogen from the anaerobic atmosphere. However, in TAF microcosms there seemed to be a connection between toluene degradation and PCE transformation (Fig. 50), and it is possible that toluene or some of its degradation products served as the electron donors for PCE reduction (although, with the data available, a mere coincidence of these two processes can not be excluded either). Possible reasons for the very low PCE transformation rate in the TAF microcosms might be the following: lower initial biomass (only 15 g of aquifer solids per 120 mL of medium were used to set up these microcosms, vs. 50-75 g of aquifer solids per 100 mL of medium for microcosms from the other two sources); lower incubation temperature (20-25°C) than in our other

experiments; and/or the slow degradation of the electron donor (toluene), on which the PCE dechlorination was dependent (Fig. 50).

PCE and CT transformations were very likely catalyzed by two different groups of microorganisms. There were several peculiarities of PCE dechlorination that were not observed with CT as a substrate. Neither PCE, nor its dechlorination products inhibited toluene degradation (Tables 16, 17; Fig. 52B); PCE transformation did not inhibit methanogenesis, but rather appeared to stimulate it (Table 14); suspended toluene-degrading methanogenic consortia did not degrade PCE at all; and, in one case, PCE reduction could probably be coupled to toluene degradation (Fig. 50). It is not clear from our results which physiological group of microorganisms was responsible for the dechlorination of PCE. Fathepure and Boyd (1988 a, b) had shown before that methanogens, such as *Methanosarcina* sp., could reductively dechlorinate PCE to TCE.

The results obtained with benzoate support the observation that CT and PCE were reductively dechlorinated. Benzoate was slowly degraded in Traverse City microcosms, but not in TAF microcosms; however, it slowed down CT and PCE transformation in both. If benzoate is degraded, it should be expected that reductive dechlorination rate would decrease: benzoate transformation under anaerobic conditions starts with ring reduction (Evans, 1977), which requires a source of electrons and would therefore compete with the reductive dechlorination process. If benzoate is not degraded (or the degradation is so slow that it can not be measured), the effect on the transformation of chlorinated aliphatics becomes less clear.

Abiotic transformation of CT, and to a much lesser degree PCE, occurred in the autoclaved controls. (An exception were the autoclaved TAF controls that did not transform PCE during the incubation period studied). Even some of the transformation products (CF from CT, TCE from PCE) could be detected in Traverse City and Pensacola controls, suggesting that reductive dechlorination was occurring also under abiotic conditions. The nonbiological transformation of CT was especially pronounced in the presence of accessory organic substrates (acetate, yeast extract, peptone) in TAF microcosms; these compounds were probably used as reductants (electron donors). Various transition metals, such as Cr(II)SO<sub>4</sub>, as well as biological forms of transition metal complexes -- heme proteins, vitamin B<sub>12</sub> (cyanocobalamin), and others -- had been reported to reductively dechlorinate CT in the presence of reducing

agents (Castro and Cray, 1963; Wood et al., 1968; Wade and Castro, 1973; Klecka and Gonsior, 1984). Similar results were reported for PCE (Gantzer and Wackett, 1991; Schanke and Wackett, 1992). Kriegman and Reinhard (1990) observed CT dechlorination to CF and other (unidentified) products with Fe(II)-containing mineral systems (sheet silicates such as biotite and vermiculite) in the presence of sulfide, and with iron sulfide minerals (pyrite, marcasite). The dechlorination rates in heterogeneous systems (containing mineral solids) were found to be much higher than rates in homogenous solutions with soluble environmental reductants (Reinhard et al., 1990). For example, half-life for CT in the presence of pyrite or marcasite was shorter than one day (Kriegman and Reinhard, 1990). In our autoclaved controls, organic reductants, microbial transition metal complexes remaining after autoclaving, and mineral surfaces could all have contributed to abiotic dechlorination of CT and PCE. However, the comparison between the active microcosms and controls indicated that the transformation in active cultures was faster and more complete than the transformation in the absence of microorganisms (Figs. 45-47, 49, 50). This corresponds to the results reported by Bouwer and McCarty (1983), Parsons and Lage (1985), and Gälli and McCarty (1989). It needs to be noted, though, that the transformation of CT in active microcosms might have been due also to chemical reactions caused by redox changes or microbial metabolites, rather than direct microbial catalysis.

Autoclaved controls revealed that PCE was sorbing to aquifer solids; instantaneous sorption was followed by slow desorption (Traverse City and Pensacola microcosms; Fig. 45A, Table 12). Sorption is probably the result of low PCE solubility in water ( $200 \text{ mg L}^{-1}$ ; Chiou et al., 1979), the corresponding moderately high octanol/water partition coefficient (Leo, 1983), and a moderately high soil/water partition coefficient (Mallon, 1989). Roberts et al. (1986) reported that sorption of PCE to aquifer solids was the reason for retardation of PCE relative to chloride tracer in a field-injection experiment in Borden aquifer (Canada). Pignatello (1990) indicated slowly reversible sorption of PCE to soils. The sorption phenomenon can help explain the unusual behavior of PCE and its dechlorination products in the active Pensacola microcosms (Fig. 48). In these microcosms, two spikes of PCE were completely removed during 110 days of incubation, but the dechlorination products (indicating biological transformation) first appeared only after 120 days. Sorption to aquifer solids and to biomass, combined with slow uptake by the biomass but no immediate



transformation, might be the reasons for a delay between PCE disappearance and the appearance of TCE and DCE.

It should be stressed that we observed only one possible case of coupling of an aromatic hydrocarbon (toluene) degradation with a chlorinated aliphatic transformation (PCE dechlorination in TAF microcosms) under methanogenic conditions. Simultaneous oxidation of aromatic hydrocarbons and reduction of chlorinated aliphatic solvents might be occurring at some sites contaminated by mixtures of these compounds, under specific conditions, but not at other sites and under other conditions. It might even be quite possible that, in some cases, chloroaliphatics would temporarily inhibit the transformation of aromatic hydrocarbons, as we found to be the case with CT. It is interesting to note that we did not observe any connection between toluene degradation and PCE dechlorination in Traverse City microcosms, which were derived from the same source as the microcosms described by Sewell and Gibson (1991), that had been shown to support the coupling of these two processes successfully. Probable reasons for this discrepancy lie in possible differences in experimental set-ups, as well as in the spatial and temporal heterogeneity in distribution of microflora within ground water aquifers; it should not be surprising that some type of biotransformation would occur in some microniches, and not in the others, within the same aquifer.

#### 4. CONCLUSIONS

The anaerobic biodegradability of representative mononuclear aromatic hydrocarbons (MAH), chlorinated benzenes (CB), and mixtures of MAH and chlorinated aliphatic solvents was studied under anaerobic conditions, with environmental samples (aquifer solids) derived from five contaminated sites. For comparison, a sewage sludge-derived, stabilized mixed methanogenic culture inoculum, enriched on an aromatic lignin derivative (ferulate) was also used. In most of the experiments, mixtures of the aromatic compounds, rather than single substrates, were used in order to address the contamination complexity common in most of the affected sites.

Some of the MAH were completely mineralized to  $\text{CO}_2$  and  $\text{CH}_4$  under methanogenic conditions, using the aquifer material from the methanogenic zone of Pensacola (FL) site that has been contaminated by creosote and pentachlorophenol for about 80 years. The mixture of the substituted MAH contaminants used in the experiments included toluene, ethylbenzene, *o*-xylene, and *p*-xylene. The only hydrocarbons degraded were toluene and *o*-xylene, indicating microbial selectivity for certain MAH isomers (e.g., *p*-xylene was not degraded, whereas *o*-xylene was). The acclimation period before the onset of biodegradation was long, on the order of a hundred days, which is most likely due to low microbial numbers on the aquifer solids ( $10^5$  to  $10^7$  total bacteria per 100 g of dry aquifer material, as determined by acridine orange direct counting; Godsy et al., 1989). The addition of *p*-cresol, a contaminant found in Pensacola site and known to be biodegraded *in situ* (Godsy et al., 1989), and also known to be a potential intermediate in methanogenic degradation of toluene (Vogel and Grbić-Galić, 1986), shortened the acclimation lag by supporting faster microbial growth. This indicates that some components of a contaminant mixture may have a beneficial effect on microbial degradation of other components.

It is important to point out that some of the replicate microcosms degraded the hydrocarbons, whereas the other replicates from the same group did not; this stresses the importance of spatial heterogeneity in microbial distribution (even on the scale of microhabitats) within the site. The microorganisms on Pensacola aquifer solids used in this study were obviously completely adapted to (and selected by) fermentative/methanogenic conditions, because -- contrary to the expectations -- the addition of exogenous electron acceptors (nitrate, sulfate, and especially oxygen) slowed down

the degradation of toluene. The studies with microbial enrichments from the microcosms yielded several notable observations. First, solid support is clearly important for microbial growth and pollutant degradation, not only because of the higher availability of nutrients at the solid/liquid interface, but also because of the necessity of "clustering" the bacteria together to facilitate the interspecies transfer of intermediates. The failure to isolate pure cultures from the enrichments underscores the importance of tight connections between the members of the community. Second, the acclimation to the pollutant substrates clearly accelerates their biodegradation, and enables microorganisms to tolerate significantly higher initial concentrations of MAH. Third, the optimal pH for degradation is the one encountered in the site (pH 6); increasing the pH strongly reduces the activity. Fourth, some MAH, although structurally different, are not necessarily degraded in sequence, but can be degraded simultaneously (toluene and *o*-xylene). Fifth, accessory organic compounds, such as acetate, methanol, propionate, fatty acids, glucose, casamino acids, pepton, yeast extract, or acetone may be preferentially used by the microorganisms and therefore slow down or inhibit MAH degradation. This finding has important implications on the fate of MAH in contaminated sites.

The aquifer solids from Seal Beach (CA) site (sulfidogenic) were used to study the degradation of MAH under sulfate-reducing conditions. The mixture of contaminants used included benzene, toluene, ethylbenzene, *o*-xylene, and *p*-xylene. Only benzene and ethylbenzene were not degraded, whereas all the other MAH were. The lag before the onset of degradation was between 40 days for toluene, to 104 days for *o*-xylene. The Seal Beach site is contaminated by gasoline; the exposure of the indigenous microorganisms to MAH obviously increased their capabilities to attack a broader range of MAH, and to do it faster (shorter "acclimation lag") than the methanogenic communities in the Pensacola site. The pollutants were degraded sequentially, not simultaneously: toluene first, *p*-xylene second, and *o*-xylene last. The enrichments derived from the sulfate-reducing microcosms were also capable to degrade *m*-xylene without a lag, although the microcosms were not originally spiked with this compound. The addition of preferential microbial substrates (glucose, lactate, yeast extract) inhibited the degradation of aromatics, which supports the conclusion that the hydrocarbons were degraded as primary substrates, but also indicates that in the sites where the concentration of natural organic carbon sources is high, the breakdown of aromatic hydrocarbons may become questionable. Environmental factors, such as pH and the medium chemistry (e.g., the presence of free sulfide)

strongly influenced the transformation process. High concentrations of sulfide (5 mM) completely inhibited the degradation, and so did the concentrations of the aromatic hydrocarbon substrates that were above 0.5 mM.

Benzene was not degraded in Seal Beach microcosms when added in mixture with other MAH; however, it was degraded by the microcosms (and enrichment cultures derived thereof) when added alone or in the presence of toluene only. This sheds some light on the frequently observed recalcitrance of benzene in natural anaerobic habitats: preferential utilization of natural organics, as well as other MAH in mixtures may completely preclude the degradation of benzene. A very long time may be necessary for the benzene degradation to start happening under natural conditions.

CB (HCB, PeCB, TTCB, TCB, DCB, MCB) were transformed in methanogenic microcosms from the Pensacola aquifer. All the CB except MCB were reductively dechlorinated. The success of the methanogenic microcosms in dechlorination of a variety of CB may be partially due to the fact that these microorganisms were adapted to the presence of pentachlorophenol (and capable of transforming it [E.M. Godsy, personal communication]) *in situ*.

Highly chlorinated CB (HCB, PeCB), and to a lesser degree TTCB and TCB were also dechlorinated under abiotic conditions, in the autoclaved controls, but at rates about one to two orders of magnitude lower than in the presence of microorganisms. Presumably, these abiotic transformations were due to thermostable microbial compounds and/or presence of various reducing inorganic species and minerals (aquifer solids) as catalysts, because no transformation was observed in sterile chemical controls without aquifer material. The dechlorinating microbial communities from the methanogenic Pensacola site exhibited acclimation to CB, which resulted in a substantial increase of dechlorination rates upon refeeding the CB to microcosms or enrichments derived from the microcosms. The addition of 2-bromoethane sulfonic acid (BESA), an inhibitor of methanogenesis, decreased the rates of reductive dechlorination, suggesting that methanogenic communities were indeed involved in the process. A specific dechlorination pattern was observed -- a preferential removal of a chlorine substituent positioned between two other chlorine substituents. The same had been observed previously by Fathepure et al. (1988) with the inoculum derived from sewage sludge, indicating that there may be some

preferential dechlorination pathways regardless of the inoculum source; this increases the capability to predict the types of products formed from the parent CB compounds in the environment.

Cross-acclimation studies with the enrichments suggested that the PeCB-dechlorinating community may be structurally different from the communities enriched on other CB. The addition of accessory organic electron donors (acetate, methanol, pepton, yeast extract, benzoate, phenol, *p*-cresol, toluene) either increased the rates of reductive dechlorination (methanol, pepton and yeast extract in some cases, acetate in rare cases [PeCB dechlorination to TTCB]), or did not influence it at all (toluene, phenol, *p*-cresol, benzoate), or decreased the rates (acetate in most cases, pepton and yeast extract in rare cases [PeCB dechlorination to TTCB]). Furthermore, the presence of CB (except MCB) inhibited the degradation of toluene which was otherwise readily degraded in similar microcosms. This underscores the importance of the types of organic compounds (either natural, or the introduced pollutants) present in the subsurface, as factors which can significantly influence the fate of CB. These results also suggest that the pollutant mixtures containing aromatic hydrocarbons and CB may be quite recalcitrant, because MAH do not represent suitable electron donors for the reductive dechlorination of CB, and CB -- in return -- may inhibit microorganisms that degrade MAH. The microcosms and enrichments that we tested also transformed CB in the absence of any additional electron donors; the two possible sources of electrons were unidentified organics on aquifer solids, and H<sub>2</sub> in the anaerobic atmosphere. However, in addition to this, poly-beta-hydroxybutyrate accumulations were observed in the microbial cells. Our previous work with aquifer microorganisms of different types (Henry and Grbić-Galić, 1990) had shown that internal storage granules are frequently accumulated by microorganisms in the subsurface; these internal reserves may be very important *in situ* for microbial processes which require reducing power.

Transformation of carbon tetrachloride (CT) and tetrachloroethylene (PCE) was studied under methanogenic conditions, in the presence or absence of several aromatic compounds (toluene, ethylbenzene, phenol, and benzoate). Microbial inocula for the experiments were derived from three ground water aquifers, contaminated by jet fuel or creosote. The results indicated that CT and PCE were reductively dechlorinated in all the examined cases (CT to chloroform [CF], and PCE to trichloroethylene [TCE], *trans*-1,2-dichloroethylene [DCE], and vinyl chloride [VC]).

The electron donors used for the reductive transformation were the unidentified organic compounds present on aquifer solids, and/or  $H_2$  from the anaerobic headspace. The addition of acetate and complex organic compounds, such as yeast extract and peptone, increased the reductive dechlorination rates. The addition of benzoate caused a decrease in rates of dechlorination, possibly because benzoate (which is initially reductively transformed under anaerobic conditions) was competing for the same sources of electrons. Phenol and ethylbenzene were not degraded, and their presence did not influence the transformation of CT or PCE. The results with toluene were mixed. This aromatic hydrocarbon was anaerobically degraded (to  $CO_2$  and  $CH_4$ ) in some of the microcosms, and in a stable methanogenic consortium derived from the microcosms. CF, a product of CT dechlorination, inhibited the toluene degradation, whereas TCE, DCE, and VC did not. In most of the studied cases, the presence of toluene had no influence on reductive dechlorination of either CT, or PCE. Only in one case (microcosms derived from a JP-4 jet fuel contaminated aquifer), a connection could possibly be established between anaerobic degradation of toluene and simultaneous reductive dechlorination of PCE, suggesting that toluene might be used as an electron donor for reductive transformation of chlorinated solvents. This phenomenon could be very important in contaminated ground water aquifers *in situ*; however, its occurrence would depend on the distribution of microorganisms within an aquifer, microbial community structure, and various environmental conditions.

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## 7. PROFESSIONAL PERSONNEL

**Li-Nuo Liang**, Research Scientist. Left Stanford in 1991. Currently at Institute of Pathology, School of Medicine, Case Western Reserve University, Cleveland, Ohio.

**Elizabeth A. Edwards**, Ph.D. candidate. Will defend her thesis in early 1993. The thesis title: "Transformation of Non-Oxygenated Homocyclic Aromatic Compounds by Aquifer Microorganisms under Sulphate-Reducing and Methanogenic Conditions".

## 8. PRESENTATIONS AT MEETINGS AND CONFERENCES

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